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Richard Zimmermann

APPLICATION FOR UNITED STATES LETTERS PATENT

S P E C I F I C A T I O N

TO ALL WHOM IT MAY CONCERN:

Be it known that we, David E. Lowery a citizen of the United States of America, residing at 1207 Woodland Drive, Portage, 49024 in the County of Kalamazoo and State of Michigan and Troy E. Fuller a citizen of the United States of America, residing at 111 Dreamfield Drive, Battle Creek, 49014, in the County of Calhoun and State of Michigan and Michael J. Kennedy a citizen of the United States of America, residing at 2364 Quincy Avenue, Portage, 49024, in the County of Kalamazoo and State of Michigan have invented a new and useful ANTI-BACTERIAL VACCINE COMPOSITIONS, of which the following is a specification.

New Patent Application for: David E. Lowery, Troy E. Fuller, and Michael J. Kennedy

For: ANTI-BACTERIAL VACCINE COMPOSITIONS

Mailing Certification for: New Patent Application

Attorney Docket No: 28341/6227.1NCP

"EXPRESS MAIL" mailing label No. EM362733684US

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Richard Zimmermann

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Joseph A. Williams, Jr.

ANTI-BACTERIAL VACCINE COMPOSITIONS

FIELD OF THE INVENTION

The present invention relates generally to the identification of genes responsible for virulence of *Pasteurella multocida* and *Actinobacillus pleuropneumoniae* bacteria, thereby allowing for production of novel attenuated mutant strains useful in vaccines and identification of new anti-bacterial agents that target the virulence genes and their products.

BACKGROUND OF THE INVENTION

The family *Pasteurellaceae* encompasses several significant pathogens that infect a wide variety of animals. In addition to *P. multocida*, prominent members of the family include *Pasteurella haemolytica, Actinobacillus pleuropneumoniae* and *Haemophilus somnus*. *P. multocida* is a gram-negative, nonmotile coccobacillus which is found in the normal flora of many wild and domestic animals and is known to cause disease in numerous animal species worldwide [Biberstein, In M. Kilian, W. Frederickson, and E. L. Biberstein (ed.), *Haemophilus, Pasteurella, and Actinobacillus*. Academic Press, London, p. 61-73 (1981)]. The disease manifestations following infection include septicemias, bronchopneumonias, rhinitis, and wound infections [Reviewed in Shewen, *et al., In* C. L. Gyles and C. O. Thoen (ed.), Pathogenesis of Bacterial Infections in Animals. Iowa State University Press, Ames, p. 216-225 (1993), incorporated herein by reference].

Infection by *P. multocida* generally results from invasion during periods of stress, but transmission may also occur by aerosol or contact exposure, or via flea and tick vectors. In fowl, *P. multocida* infection gives rise to acute to peracute septicemia, particularly prevalent in domestic turkeys and wild waterfowl under stress conditions associated with overcrowding, laying, molting, or severe climatic change. In cattle, a similar hemorrhagic septicemia follows infection and manifests conditions including high fever and depression, generally followed by quick death. Transmission is most likely through aerosol contact, but infection can also arise during periods of significant climatic change. In rabbits, infection gives rise to recurring purulent rhinitis, generally followed by conjunctivitis, otitis media, sinusitis, subcutaneous abscesses, and chronic

bronchopneumonia. In severe infections, rabbit mortality arises from acute fibrinous bronchopneumonia, septicemia, or endotoxemia. Disease states normally arise during periods of stress. In pigs, common *P. multocida* disease states include atrophic rhinitis and bacterial pneumonia. Similar pneumonia conditions are also detected in dogs, cats, goats, and sheep. *P. multocida* is commonly detected in oral flora of many animals and is therefore a common contaminant in bite and scratch wounds.

P. multocida strains are normally designated by capsular serogroup and somatic serotype. Five capsular serogroups (A, B, D, E, and F) and 16 somatic serotypes are distinguished by expression of characteristic heat-stable antigens. Most strains are host specific and rarely infect more than one or two animals. The existence of different serotypes presents a problem for vaccination because traditional killed whole cell bacteria normally provide only serotype-specific protection. However, it has been demonstrated that natural infection with one serotype can lead to immunological protection against multiple serotypes [Shewen, *et al., In* C. L. Gyles and C. O. Thoen (Ed.), Pathogenesis of Bacterial Infections in Animals. Iowa State University Press, Ames, p. 216-225 (1993)] and cross protection can also be stimulated by using inactivated bacteria grown *in vivo* [Rimler, *et al., Am J Vet Res. 42*:2117-2121 (1981)]. One live spontaneous mutant *P. multocida* strain has been utilized as a vaccine and has been shown to stimulate a strong immune response [Davis, *Poultry Digest. 20*:430-434 (1987), Schlink, *et al., Avian Dis. 31*(1):13-21 (1987)]. This attenuated strain, however, has been shown to revert to a virulent state or cause mortality if the vaccine recipient is stressed [Davis, *Poultry Digest. 20*:430-434 (1987), Schlink, *et al., Avian Dis. 31*(1):13-21 (1987)].

Another member of the *Pasteurella* family, *A. pleuropneumoniae* exhibits strict host specificity for swine and is the causative agent of highly contagious porcine pleuropneumonia. Infection normally arises in intensive breeding conditions, and is believed to occur by a direct mode of transmission. The disease is often fatal and, as a result, leads to severe economic loss in the swine producing industry. *A. pleuropneumoniae* infection may be chronic or acute, and infection is characterized by a hemorrhagic, necrotic bronchopneumonia with accompanying fibrinous pleuritis. To date, bacterial virulence has been attributed to structural proteins, including serotype-specific capsular polysaccharides, lipopolysaccharides, and surface proteins, as well as

extracellular cytolytic toxins. Despite purification and, in some instances cloning, of these virulence factors, the exact role of these virulence factors in *A. pleuropneumoniae* infection is poorly understood.

Twelve serotypes of *A. pleuropneumoniae* have been identified based on antigenic differences in capsular polysaccharides and production of extracellular toxins. Serotypes 1, 5, and 7 are most relevant to *A. pleuropneumoniae* infection in the United States, while serotypes 1, 2, 5, 7, and 9 are predominant in Europe. There are at least three significant extracellular toxins of *A. pleuropneumoniae* that are members of the haemolysin family and are referred to as RTX toxins. RTX toxins are produced by many Gram negative bacteria, including *E. coli*, *Proteus vulgarisa*, and *Pasteurella haemolytica*, and the proteins generally share structural and functional characteristics. Toxins from the various serotypes differ, however, in host specificity, target cells, and biological activities.

The major *A. pleuropneumoniae* RTX toxins include ApxI, ApxII, and ApxIII. ApxI and ApxII have haemolytic activity, with ApxI being more potent. ApxIII shows no haemolytic activity, but is cytotoxic for alveolar macrophages and neutrophils. Most *A. pleuropneumoniae* serotypes produce two of these three toxins. For example, serotypes 1, 5, 9, and 11 express ApxI and ApxII, and serotypes 2, 3, 4, 6, and 8 express ApxII and ApxIII. Serotype 10, however, produces only ApxI, and serotypes 7 and 12 express only ApxII. Those *A. pleuropneumoniae* serotypes that produce both ApxI and ApxII are the most virulent strains of the bacteria.

The Apx toxins were demonstrated to be virulence factors in murine models and swine infection using randomly mutated wild type bacteria [Tascon, *et al., Mol. Microbiol. 14*:207-216 (1994)]. Other *A. pleuropneumoniae* mutants have also been generated with targeted mutagenesis to inactivate the gene encoding the AopA outer membrane virulence protein [Mulks and Buysee, *Gene 165*:61-66 (1995)].

In attempts to produce vaccine compositions, traditional killed whole cell bacteria have provided only serotype-specific protection [MacInnes and Smart, *supra*], however, it has been demonstrated that natural infection with a highly virulent serotype can stimulate strong protective immunity against multiple serotypes [Nielsen, *Nord Vet Med. 31*:407-13 (1979), Nielsen, *Nord Vet Med. 36*:221-234 (1984), Nielsen, *Can J Vet*

Res. 29:580-582 (1988), Nielsen, *ACTA Vet Scand. 15*:80-89 (1994)]. One defined live-attenuated vaccine strain producing an inactive form of the ApxII toxin has shown promise for cross protection in swine [Prideaux, *et al., Infection & Immunity 67*:1962-1966 (1999)], while other undefined live-attenuated mutants have also shown promise [Inzana, *et al., Infect Immun. 61*:1682-6, (1993), Paltineanu, et al., *In* International Pig Veterinary Society, 1992, p. 214, Utrera, *et al., In* International Pig Veterinary Society, 1992, p. 213].

Because of the problems associated with vaccine formulations comprising bacterial strains with undefined, spontaneous mutations, there exists a need in the art for rational construction of live attenuated bacterial strains for use in vaccines that will safely stimulate protective immunity against homologous and heterologous *P. multocida* and *A. pleuropneumoniae* serotypes. There further exists a need to identify attenuated bacterial strains and genes required for bacterial virulence, thereby facilitating development of methods to identify anti-bacterial agents.

SUMMARY OF THE INVENTION

In general, the present invention provides materials and methods for production and use of vaccine compositions comprising attenuated gram negative bacteria. In one aspect, vaccine compositions of the invention comprise attenuated species in the *Pasteurellaceae* family of bacteria, which is known in the art and described, in part, in Dewhirst, *et al., J. Bacteriol. 174*:2002-2013 (1992), incorporated herein by reference in its entirety. Species in the family include, but are not limited to, *A. actinomycetemcomitans, A. capsulatus, A. equuli, A. lignieresii, A. pleuropneumoniae (H. pleuropneumoniae), A. seminis, A. suis (H. suis), A. ureae (p. ureae), A. capsulatus,* Bisgaard taxon 11, *H. aegyptius, H. aphrophilus, H. aphrophilus (H. parainfluenzae), H. ducreyi, H.* haemoglobinophilus, *H. haemolyticus, H. influenzae, H. paracuniculus, H. paragallinarum, H. parahaemolyticus, H. parainfluenzae,* (*H. paraphrophilus*), *H. paraphrohaemolyticus, H. paraphrophilus, H. parasuis, H. parasuis* type 5, *H. segnis, H. somnus, Haemophilus* minor group, *Haemophilus* taxon C, *P. aerogenes, P. anatis, P. avium (H. avium), P. canis, P. dagmatis, P. gallinarum, P. haemolytica, P. trehalosi* (*P. haemolytica* biotype T), *P. langaa, P. multocida, P. pneumotropica, P. stomatis, P.*

volantium (H. parainfluenzae), *P. volantium*, *Pasteurella* species A, *Pasteurella* species B, and *Haemophilus paraphrohaemolyticus*. Preferably, vaccine compositions comprise attenuated *Pasteurella haemolytica, Actinobacillus pleuropneumoniae, Haemophilus somnus*, or *Pasteurella multocida* bacteria. In a most preferred embodiment, vaccine compositions of the invention comprise attenuated *Pasteurella multocida* and *A. plueropneumoniae* bacterial strains.

One aspect of the invention provides gram negative bacterial organisms containing a functional mutation in a gene sequence represented by any one of SEQ ID NOS: 1, 3, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 29, 31, 33, 37, 39, 41, 51, 53, 55, 57, 58, 60, 68, 70, 72, 74, 76, 78, 80, 82, 84, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 135, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 163, and 164, or species homologs thereof, wherein the mutation inhibits or abolishes expression and/or biological activity of an encoded gene product (*i.e.*, the polypeptide encoded by a gene); said functional mutation resulting in attenuated virulence of the bacterial strain. As understood in the art, species homologs include genes found in two or more different species which possess substantial polynucleotide sequence homology and possess the same, or similar, biological functions and/or properties. Preferably polynucleotide sequences which represent species homologs will hybridize under moderately stringent conditions, as described herein by example, and possess the same or similar biological activities and or properties. In another aspect, polynucleotides representing species homologs will share greater than about 60% sequence homology, greater than about 70% sequence homology, greater than about 80% sequence homology, greater than about 90% sequence homology or greater than about 95% sequence homology. Functional mutations that modulate (*i.e.*, increase or decrease) expression and/or biological activity of a gene product include insertions or deletions in the protein coding region of the gene itself or in sequences responsible for, or involved in, control of gene expression. Deletion mutants include those wherein all or part of a specific gene sequence is deleted. In one aspect, the mutation results in deletion of at least about 10%, at least about 20%, at least about 30%, at least about 40% at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95%, at least about 98%, or at least about 99% of said gene. In another

aspect, the mutation results in an insertion in the gene, wherein the insertion causes decreased expression of a gene product encoded by the mutated gene and/or expression of an inactive gene product encoded by the mutated gene. Also contemplated are compositions, and preferably vaccine compositions, comprising mutated and attenuated gram negative bacterial organisms, optionally comprising a suitable adjuvant and/or a pharmaceutically acceptable diluent or carrier. In order for a modified strain to be effective in a vaccine formulation, the attenuation must be significant enough to prevent the pathogen from evoking severe clinical symptoms, but also insignificant enough to allow limited replication and growth of the bacteria in the host.

The invention also provides polynucleotides encoding gene products that are required for virulence in gram negative bacteria. Polynucleotides of the invention include DNA, such as complementary DNA, genomic DNA including complementary or anti-sense DNA, and wholly or partially synthesized DNA; RNA, including sense and antisense strands; and peptide nucleic acids as described, for example in Corey, *TIBTECH 15*:224-229 (1997). Virulence gene polynucleotides of the invention include those set forth in SEQ ID NOs:1, 3, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 29, 31, 33, 37, 39, 41, 51, 53, 55, 57, 58, 60, 68, 70, 72, 74, 76, 78, 80, 82, 84, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 135, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 163, and 164, or species homologs thereof, polynucleotides encoding a virulence gene product encoded by a polynucleotide of SEQ ID NOs: 1, 3, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 29, 31, 33, 37, 39, 41, 51, 53, 55, 57, 58, 60, 68, 70, 72, 74, 76, 78, 80, 82, 84, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 135, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 163, and 164, or a species homolog thereof, and polynucleotide that hybridize, under moderately to highly stringent conditions, to the noncoding strand (or complement) of any one of the polynucleotides set out in SEQ ID NOs: 1, 3, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 29, 31, 33, 37, 39, 41, 51, 53, 55, 57, 58, 60, 68, 70, 72, 74, 76, 78, 80, 82, 84, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 135, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 163, and 164, species homologs thereof. The invention therefore comprehends gene sequences from *Pasteurellaceae* set out in SEQ ID NOs: 1,

3, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 29, 31, 33, 37, 39, 41, 51, 53, 55, 57, 58, 60, 68, 70, 72, 74, 76, 78, 80, 82, 84, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 135, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 163, and 164, as well as related gene sequences from other gram negative bacterial organisms, including naturally occurring (*i.e.*, species homologs) and artificially induced variants thereof. The invention also comprehends polynucleotides which encode polypeptides deduced from any one of the polynucleotides set out in SEQ ID NOs: 1, 3, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 29, 31, 33, 37, 39, 41, 51, 53, 55, 57, 58, 60, 68, 70, 72, 74, 76, 78, 80, 82, 84, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 135, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, and 164, and species homologs thereof. Knowledge of the sequence of a polynucleotide of the invention makes readily available every possible fragment of that polynucleotide. The invention therefore provides fragments of a polynucleotide of the invention.

The invention further embraces expression constructs comprising polynucleotides of the invention. Host cells transformed, transfected or electroporated with a polynucleotide of the invention are also contemplated. The invention provides methods to produce a polypeptide encoded by a polynucleotide of the invention comprising the steps of growing a host cell of the invention under conditions that permit, and preferably promote, expression of a gene product encoded by the polynucleotide, and isolating the gene product from the host cell or the medium of its growth.

Identification of polynucleotides of the invention makes available the encoded polypeptides. Polypeptides of the invention include full length and fragment, or truncated, proteins; variants thereof; fusion, or chimeric proteins; and analogs, including those wherein conservative amino acid substitutions have been introduced into wild-type polypeptides. Antibodies that specifically recognize polypeptides of the invention are also provided, and include monoclonal and polyclonal antibodies, single chain antibodies, chimeric antibodies, humanized antibodies, human antibodies, and complementary determining region (CDR)-grafted antibodies, as well as compounds that include CDR sequences which specifically recognize a polypeptide of the invention. The

invention also provides anti-idiotype antibodies immunospecific for antibodies of the invention.

According to another aspect of the invention, methods are provided for identifying novel anti-bacterial agents that modulate the function of gram negative bacteria virulence genes or gene products. Methods of the invention include screening potential agents for the ability to interfere with expression of virulence gene products encoded by the DNA sequences set forth in any one of SEQ ID NOS: 1, 3, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 29, 31, 33, 37, 39, 41, 51, 53, 55, 57, 58, 60, 68, 70, 72, 74, 76, 78, 80, 82, 84, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 135, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 163, and 164, or species homologs thereof, or screening potential agents for the ability to interfere with biological function of a bacterial gene product encoded in whole or in part by a DNA sequence set forth in any one of SEQ ID NOS: 1, 3, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 29, 31, 33, 37, 39, 41, 51, 53, 55, 57, 58, 60, 68, 70, 72, 74, 76, 78, 80, 82, 84, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 135, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 163, and 164, species homologs thereof, or the complementary strand thereof, followed by identifying agents that provide positive results in such screening assays. In particular, agents that interfere with the expression of virulence gene products include anti-sense polynucleotides and ribozymes that are complementary to the virulence gene sequences. The invention further embraces methods to modulate transcription of gene products of the invention through use of oligonucleotide-directed triplet helix formation.

Agents that interfere with the function of virulence gene products include variants of virulence gene products, binding partners of the virulence gene products and variants of such binding partners, and enzyme inhibitors (where the product is an enzyme).

Novel anti-bacterial agents identified by the methods described herein are provided, as well as methods for treating a subject suffering from infection with gram negative bacteria involving administration of such novel anti-bacterial agents in an amount effective to reduce bacterial presence.

Numerous additional aspects and advantages of the invention will become apparent to those skilled in the art upon consideration of the following detailed description of the invention which describes presently prepared embodiments thereof.

DETAILED DESCRIPTION OF THE INVENTION

"Virulence genes," as used herein, are genes whose function or products are required for successful establishment and/or maintenance of bacterial infection in a host animal. Thus, virulence genes and/or the proteins encoded thereby are involved in pathogenesis in the host organism, but may not be necessary for growth.

"Signature-tagged mutagenesis (STM)," as used herein, is a method generally described in International Patent Publication No. WO 96/17951, incorporated herein by reference, and includes, for example, a method for identifying bacterial genes required for virulence in a murine model of bacteremia. In this method, bacterial strains that each have a random mutation in the genome are produced using transposon integration; each insertional mutation carries a different DNA signature tag which allows mutants to be differentiated from each other. The tags comprise 40 bp variable central regions flanked by invariant "arms" of 20 bp which allow the central portions to be co-amplified by polymerase chain reaction (PCR). Tagged mutant strains are assembled in microtiter dishes, then combined to form the "inoculum pool" for infection studies. At an appropriate time after inoculation, bacteria are isolated from the animal and pooled to form the "recovered pool." The tags in the recovered pool and the tags in the inoculum pool are separately amplified, labeled, and then used to probe filters arrayed with all of the different tags representing the mutants in the inoculum. Mutant strains with attenuated virulence are those which cannot be recovered from the infected animal, *i.e.*, strains with tags that give hybridization signals when probed with tags from the inoculum pool but not when probed with tags from the recovered pool. In a variation of this method, non-radioactive detection methods such as chemiluminescence can be used

Signature-tagged mutagenesis allows a large number of insertional mutant strains to be screened simultaneously in a single animal for loss of virulence. Screening nineteen pools of mutant *P. multocida* strains resulted in the identification of more than 60 strains with reduced virulence, many of which were confirmed to be attenuated in

virulence by subsequent determination of an approximate LD_{50} for the individual mutants. Screening of *A. pleuropneumoniae* mutants resulted in identification of more than 100 strains having mutations in 35 different genes. Of these, mutations in 22 genes results in significantly attenuated *A. pleuropneumoniae* strains. The nucleotide sequence of the open reading frame disrupted by the transposon insertion was determined by sequencing both strands and an encoded amino acid sequence was deduced. Novelty of both the polynucleotide and amino acid sequences was determined by comparison of the sequences with DNA and protein database sequences.

The identification of bacterial, and more particularly *P. multocida* and *A. pleuropneumoniae* virulence genes provides for microorganisms exhibiting reduced virulence (*i.e.*, attenuated strains), which are useful in vaccines. Such microorganisms include *Pasteurellaceae* mutants containing at least one functional mutation inactivating a gene represented by any one of SEQ ID NOS: 1, 3, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 29, 31, 33, 37, 39, 41, 51, 53, 55, 57, 58, 60, 68, 70, 72, 74, 76, 78, 80, 82, 84, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 135, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 163, and 164. The worker of ordinary skill in the art will realize that a "functional mutation" may occur in protein coding regions of a gene of the invention, as well as in regulatory regions that modulate transcription of the virulence gene RNA.

The worker of ordinary skill will also appreciate that attenuated *P. multocida* and *A. pleuropneumoniae* strains of the invention include those bearing more than one functional mutation. More than one mutation may result in additive or synergistic degrees of attenuation. Multiple mutations can be prepared by design or may fortuitously arise from a deletion event originally intended to introduce a single mutation. An example of an attenuated strain with multiple deletions is a *Salmonella typhimurium* strain wherein the *cya* and *crp* genes are functionally deleted. This mutant *S. typhimurium* strain has shown promise as a live vaccine.

Identification of virulence genes in *P. multocida* and *A. pleuropneumoniae* can provide information regarding similar genes, *i.e.*, species homologs, in other pathogenic species. As an example, identification of the *aroA* gene led to identification of conserved genes in a diverse number of pathogens, including *P. haemolytica*,

Aeromonas hydrophila, Aeromonas salmonicida, Salmonella typhimurium, Salmonella enteritidis, Salmonella dublin, Salmonella gallanerum, Bordella pertussis, Yersinia entericolitica, Neisseria gonorrhoeae, and Bacillus anthracis. In many of these species, attenuated bacterial strains bearing mutations in the *aroA* gene have proven to be effective in vaccine formulations. Using the virulence genes sequences identified in *P. multocida*, similar or homologous genes can be identified in other organisms, particularly within the *Pasteurella* family, as well as *A. pleuropneumoniae* and *Haemophilus somnus*. Likewise, identification of *A. pleuropneumoniae* virulence genes can permit identification of related genes in other organisms. Southern hybridization using the *P. multocida* and *A. pleuropneumoniae* genes as probes can identify these related genes in chromosomal libraries derived from other organisms. Alternatively, PCR can be equally effective in gene identification across species boundaries. As still another alternative, complementation of, for example, a *P. multocida* mutant with a chromosomal library from other species can also be used to identify genes having the same or related virulence activity. Identification of related virulence genes can therefore lead to production of an attenuated strain of the other organism which can be useful as still another vaccine formulation. Examples of *P. multocida* genes that have been demonstrated to exist in other species (*e.g. P. haemolytica, A. pleuropneumoniae* and *H. somnus*) include genes *exbB, atpG, and pnp*

Attenuated *P. multocida* strains identified using STM are insertional mutants wherein a virulence gene has been rendered non-functional through insertion of transposon sequences in either the open reading frame or regulatory DNA sequences. In one aspect, therefore, the attenuated *P. multocida* strains, as well as other gram-negative mutant bacterial strains of the invention can bear one or more mutations which result in an insertion in the gene, with the insertion causing decreased expression of a gene product encoded by the mutated gene and/or expression of an inactive gene product encoded by the mutated gene. These insertional mutants still contain all of the genetic information required for bacterial virulence and can possibly revert to a pathogenic state by deletion of the inserted transposon. Therefore, in preparing a vaccine formulation, it is desirable to take the information gleaned from the attenuated strain and create a deletion mutant strain wherein some, most, or all of the virulence gene sequence is

removed, thereby precluding the possibility that the bacteria will revert to a virulent state. The attenuated *P. multocida* strains, as well as other gram-negative mutant bacterial strains of the invention therefore include those bearing one or more mutation which results in deletion of at least about 10%, at least about 20%, at least about 30%, at least about 40% at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95%, at least about 98%, or at least about 99% of the virulence gene.

The vaccine properties of an attenuated insertional mutant identified using STM are expected to be the same or similar to those of a bacteria bearing a deletion in the same gene. However, it is possible that an insertion mutation may exert "polar" effects on adjoining gene sequences, and as a result, the insertion mutant may possess characteristic distinct from a mutant strain with a deletion in the same gene sequence. Deletion mutants can be constructed using any of a number of techniques well known and routinely practiced in the art.

In one example, a strategy using counterselectable markers can be employed which has commonly been utilized to delete genes in many bacteria. For a review, see, for example, Reyrat, *et al., Infection and Immunity 66*:4011-4017 (1998), incorporated herein by reference. In this technique, a double selection strategy is often employed wherein a plasmid is constructed encoding both a selectable and counterselectable marker, with flanking DNA sequences derived from both sides of the desired deletion. The selectable marker is used to select for bacteria in which the plasmid has integrated into the genome in the appropriate location and manner. The counterselecteable marker is used to select for the very small percentage of bacteria that have spontaneously eliminated the integrated plasmid. A fraction of these bacteria will then contain only the desired deletion with no other foreign DNA present. The key to the use of this technique is the availability of a suitable counterselectable marker.

In another technique, the *cre-lox* system is used for site specific recombination of DNA. The system consists of 34 base pair *lox* sequences that are recognized by the bacterial *cre* recombinase gene. If the *lox* sites are present in the DNA in an appropriate orientation, DNA flanked by the *lox* sites will be excised by the *cre* recombinase, resulting in the deletion of all sequences except for one remaining copy of

the *lox* sequence. Using standard recombination techniques, it is possible to delete the targeted gene of interest in the *P. multocida* or *A. pleuropneumoniae* genome and to replace it with a selectable marker (*e.g.*, a gene coding for kanamycin resistance) that is flanked by the *lox* sites. Transient expression (by electroporation of a suicide plasmid containing the *cre* gene under control of a promoter that functions in *P. multocida* or *A. pleuropneumoniae*) of the *cre* recombinase should result in efficient elimination of the *lox* flanked marker. This process would result in a mutant containing the desired deletion mutation and one copy of the *lox* sequences.

In another approach, it is possible to directly replace a desired deleted sequence in the *P. multocida* or *A. pleuropneumoniae* genome with a marker gene, such as green fluorescent protein (GFP), β-galactosidase, or luciferase. In this technique, DNA segments flanking a desired deletion are prepared by PCR and cloned into a suicide (non-replicating) vector for *P. multocida* or *A. pleuropneumoniae*. An expression cassette, containing a promoter active in *P. multocida* or *A. pleuropneumoniae* and the appropriate marker gene, is cloned between the flanking sequences. The plasmid is introduced into wild-type *P. multocida* or *A. pleuropneumoniae*. Bacteria that incorporate and express the marker gene (probably at a very low frequency) are isolated and examined for the appropriate recombination event (*i.e.*, replacement of the wild type gene with the marker gene).

The reduced virulence of these organisms and their immunogenicity may be confirmed by administration to a subject animal. While it is possible for an avirulent microorganism of the invention to be administered alone, one or more of such mutant microorganisms are preferably administered in a vaccine composition containing suitable adjuvant(s) and pharmaceutically acceptable diluent(s) or carrier(s). The carrier(s) must be "acceptable" in the sense of being compatible with the avirulent microorganism of the invention and not deleterious to the subject to be immunized. Typically, the carriers will be water or saline which will be sterile and pyrogen free. The subject to be immunized is a subject needing protection from a disease caused by a virulent form of *P. multocida*, *A. pleuropneumoniae*, or other pathogenic microorganisms.

It will be appreciated that the vaccine of the invention may be useful in the fields of human medicine and veterinary medicine. Thus, the subject to be

immunized may be a human or other animal, for example, farm animals including cows, sheep, pigs, horses, goats and poultry (*e.g.*, chickens, turkeys, ducks and geese) companion animals such as dogs and cats; exotic and/or zoo animals; and laboratory animals including mice, rats, rabbits, guinea pigs, and hamsters.

The invention also provides polypeptides and corresponding polynucleotides required for *P. multocida* or *A. pleuropneumoniae* virulence. The invention includes both naturally occurring and non-naturally occurring polynucleotides and polypeptide products thereof. Naturally occurring virulence products include distinct gene and polypeptide species as well as corresponding species homologs expressed in organisms other than *P. multocida* or *A. pleuropneumoniae* strains. Non-naturally occurring virulence products include variants of the naturally occurring products such as analogs and virulence products which include covalent modifications. In a preferred embodiment, the invention provides virulence polynucleotides comprising the sequences set forth in SEQ ID NOs: 1, 3, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 29, 31, 33, 37, 39, 41, 51, 53, 55, 57, 58, 60, 68, 70, 72, 74, 76, 78, 80, 82, 84, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 135, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 163, and 164, and species homologs thereof, and polypeptides having amino acids sequences encoded by the polynucleotides.

The present invention provides novel purified and isolated *P. multocida* and *A. pleuropneumoniae* polynucleotides (e.g., DNA sequences and RNA transcripts, both sense and complementary antisense strands) encoding the bacterial virulence gene products. DNA sequences of the invention include genomic and cDNA sequences as well as wholly or partially chemically synthesized DNA sequences. Genomic DNA of the invention comprises the protein coding region for a polypeptide of the invention and includes variants that may be found in other bacterial strains of the same species. "Synthesized," as used herein and is understood in the art, refers to purely chemical, as opposed to enzymatic, methods for producing polynucleotides. "Wholly" synthesized DNA sequences are therefore produced entirely by chemical means, and "partially" synthesized DNAs embrace those wherein only portions of the resulting DNA were produced by chemical means. Preferred DNA sequences encoding *P. multocida*

virulence gene products are set out in SEQ ID NOs: 1, 3, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 29, 31, 33, 37, 39, 41, 51, 53, 55, 57, 58, 60, 68, 70, 72, 74, 76, 78, 80, 82, 84, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, and 120, 122, 124, 126, 128, 130, 132, 134, 135, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 163, and 164, and species homologs thereof. Preferred *A. pleuropneumoniae* DNA sequences encoding virulence gene products are set out in SEQ ID NOs: 122, 124, 126, 128, 130, 132, 134, 135, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 163, and 164, and species homologs thereof. The worker of skill in the art will readily appreciate that the preferred DNA of the invention comprises a double stranded molecule, for example, molecules having the sequences set forth in SEQ ID NOs: 1, 3, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 29, 31, 33, 37, 39, 41, 51, 53, 55, 57, 58, 60, 68, 70, 72, 74, 76, 78, 80, 82, 84, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, and 120, 122, 124, 126, 128, 130, 132, 134, 135, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 163, and 164, and species homologs thereof, along with the complementary molecule (the "non-coding strand" or "complement") having a sequence deducible from the sequence of SEQ ID NO: 1, 3, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 29, 31, 33, 37, 39, 41, 51, 53, 55, 57, 58, 60, 68, 70, 72, 74, 76, 78, 80, 82, 84, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, and 120, 122, 124, 126, 128, 130, 132, 134, 135, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 163, and 164, according to Watson-Crick base pairing rules for DNA. Also preferred are polynucleotides encoding the gene products encoded by any one of the polynucleotides set out in SEQ ID NOs: 1, 3, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 29, 31, 33, 37, 39, 41, 51, 53, 55, 57, 58, 60, 68, 70, 72, 74, 76, 78, 80, 82, 84, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, and 120, 122, 124, 126, 128, 130, 132, 134, 135, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 163, and 164, and species homologs thereof. The invention further embraces species, preferably bacterial, homologs of the *P. multocida* and *A. pleuropneumoniae* DNA.

The polynucleotide sequence information provided by the invention makes possible the identification and isolation of polynucleotides encoding related bacterial virulence molecules by well known techniques including Southern and/or Northern hybridization, and polymerase chain reaction (PCR). Examples of related polynucleotides include polynucleotides encoding polypeptides homologous to a

virulence gene product encoded by any one of the polynucleotides set out in SEQ ID NOs: 1, 3, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 29, 31, 33, 37, 39, 41, 51, 53, 55, 57, 58, 60, 68, 70, 72, 74, 76, 78, 80, 82, 84, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, and 120, 122, 124, 126, 128, 130, 132, 134, 135, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 163, and 164, and species homologs thereof, and structurally related polypeptides sharing one or more biological and/or physical properties of a virulence gene product of the invention.

The invention also embraces DNA sequences encoding bacterial gene products which hybridize under moderately to highly stringent conditions to the non-coding strand, or complement, of any one of the polynucleotides set out in SEQ ID NOs: 1, 3, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 29, 31, 33, 37, 39, 41, 51, 53, 55, 57, 58, 60, 68, 70, 72, 74, 76, 78, 80, 82, 84, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, and 120, 122, 124, 126, 128, 130, 132, 134, 135, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 163, and 164, and species homologs thereof. DNA sequences encoding virulence polypeptides which would hybridize thereto but for the degeneracy of the genetic code are contemplated by the invention. Exemplary high stringency conditions include a final wash in buffer comprising 0.2X SSC/0.1% SDS, at 65°C to 75°C, while exemplary moderate stringency conditions include a final wash in buffer comprising 2X SSC/0.1% SDS, at 35°C to 45°C. It is understood in the art that conditions of equivalent stringency can be achieved through variation of temperature and buffer, or salt concentration as described in Ausubel, *et al.* (Eds.), Protocols in Molecular Biology, John Wiley & Sons (1994), pp. 6.0.3 to 6.4.10. Modifications in hybridization conditions can be empirically determined or precisely calculated based on the length and the percentage of guanosine/cytosine (GC) base pairing of the probe. The hybridization conditions can be calculated as described in Sambrook, *et al.*, (Eds.), Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York (1989), pp. 9.47 to 9.51.

Autonomously replicating recombinant expression constructions such as plasmid and viral DNA vectors incorporating virulence gene sequences are also provided. Expression constructs wherein virulence polypeptide-encoding polynucleotides are operatively linked to an endogenous or exogenous expression control DNA sequence and

a transcription terminator are also provided. The virulence genes may be cloned by PCR, using *P. multocida* genomic DNA as the template. For ease of inserting the gene into expression vectors, PCR primers are chosen so that the PCR-amplified gene has a restriction enzyme site at the 5′ end preceding the initiation codon ATG, and a restriction enzyme site at the 3′ end after the termination codon TAG, TGA or TAA. If desirable, the codons in the gene are changed, without changing the amino acids, according to *E. coli* codon preference described by Grosjean and Fiers, *Gene, 18*:199-209 (1982), and Konigsberg and Godson, *Proc. Natl. Acad. Sci. (USA), 80*:687-691 (1983). Optimization of codon usage may lead to an increase in the expression of the gene product when produced in *E. coli.* If the gene product is to be produced extracellularly, either in the periplasm of *E. coli* or other bacteria, or into the cell culture medium, the gene is cloned without its initiation codon and placed into an expression vector behind a signal sequence.

According to another aspect of the invention, host cells are provided, including procaryotic and eukaryotic cells, either stably or transiently transformed, transfected, or electroporated with polynucleotide sequences of the invention in a manner which permits expression of virulence polypeptides of the invention. Expression systems of the invention include bacterial, yeast, fungal, viral, invertebrate, and mammalian cells systems. Host cells of the invention are a valuable source of immunogen for development of antibodies specifically immunoreactive with the virulence gene product. Host cells of the invention are conspicuously useful in methods for large scale production of virulence polypeptides wherein the cells are grown in a suitable culture medium and the desired polypeptide products are isolated from the cells or from the medium in which the cells are grown by, for example, immunoaffinity purification or any of the multitude of purification techniques well known and routinely practiced in the art. Any suitable host cell may be used for expression of the gene product, such as *E. coli*, other bacteria, including *P. multocida, Bacillus* and *S. aureus*, yeast, including *Pichia pastoris* and *Saccharomyces cerevisiae*, insect cells, or mammalian cells, including CHO cells, utilizing suitable vectors known in the art. Proteins may be produced directly or fused to a peptide or polypeptide, and either intracellularly or extracellularly by secretion into the periplasmic space of a bacterial cell or into the cell culture medium. Secretion of a

protein requires a signal peptide (also known as pre-sequence); a number of signal sequences from prokaryotes and eukaryotes are known to function for the secretion of recombinant proteins. During the protein secretion process, the signal peptide is removed by signal peptidase to yield the mature protein.

To simplify the protein purification process, a purification tag may be added either at the 5′ or 3′ end of the gene coding sequence. Commonly used purification tags include a stretch of six histidine residues (U.S. Patent Nos. 5,284,933 and 5,310,663), a streptavidin-affinity tag described by Schmidt and Skerra, *Protein Engineering, 6*:109-122 (1993), a FLAG peptide [Hopp *et al.*, *Biotechnology, 6*:1205-1210 (1988)], glutathione S-transferase [Smith and Johnson, *Gene, 67*:31-40 (1988)], and thioredoxin [LaVallie *et al.*, *Bio/Technology, 11*:187-193 (1993)]. To remove these peptide or polypeptides, a proteolytic cleavage recognition site may be inserted at the fusion junction. Commonly used proteases are factor Xa, thrombin, and enterokinase.

The invention also provides purified and isolated *P. multocida* and *A. pleuropneumoniae* virulence polypeptides encoded by a polynucleotide of the invention. Presently preferred are polypeptides comprising the amino acid sequences encoded by any one of the polynucleotides set out in SEQ ID NOs : 1, 3, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 29, 31, 33, 37, 39, 41, 51, 53, 55, 57, 58, 60, 68, 70, 72, 74, 76, 78, 80, 82, 84, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, and 120, 122, 124, 126, 128, 130, 132, 134, 135, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, and 164, and species homologs thereof. The invention embraces virulence polypeptides encoded by a DNA selected from the group consisting of : a) the DNA sequence set out in any one of SEQ ID NOs: 1, 3, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 29, 31, 33, 37, 39, 41, 51, 53, 55, 57, 58, 60, 68, 70, 72, 74, 76, 78, 80, 82, 84, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, and 120, 122, 124, 126, 128, 130, 132, 134, 135, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, and 164, and species homologs thereof; b) DNA molecules encoding *P. multocida* or *A. pleuropneumoniae* polypeptides encoded by any one of SEQ ID NOs: 1, 3, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 29, 31, 33, 37, 39, 41, 51, 53, 55, 57, 58, 60, 68, 70, 72, 74, 76, 78, 80, 82, 84, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, and 120, 122, 124, 126, 128, 130, 132, 134, 135, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, and 164, and species homologs thereof; and

c) a DNA molecule, encoding a virulence gene product, that hybridizes under moderately stringent conditions to the DNA of (a) or (b).

The invention also embraces polypeptides, *i.e.,* species homologs and orthologs, that have at least about 99%,at least about 95%, at least about 90%, at least about 85%, at least about 80%, at least about 75%, at least about 70%, at least about 65%, at least about 60%, at least about 55%, and at least about 50% identity and/or homology to the preferred polypeptides of the invention. Percent amino acid sequence "identity" with respect to the preferred polypeptides of the invention is defined herein as the percentage of amino acid residues in the candidate sequence that are identical with the residues in the virulence gene product sequence after aligning both sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Percent sequence "homology" with respect to the preferred polypeptides of the invention is defined herein as the percentage of amino acid residues in the candidate sequence that are identical with the residues in one of the virulence polypeptide sequences after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and also considering any conservative substitutions as part of the sequence identity. Conservative substitutions can be defined as set out in Tables A and B.

Table A
Conservative Substitutions I

SIDE CHAIN CHARACTERISTIC		AMINO ACID
Aliphatic	Non-polar	G A P I L V
	Polar - uncharged	C S T M N Q
	Polar - charged	D E K R
Aromatic		H F W Y
Other		N Q D E

Polypeptides of the invention may be isolated from natural bacterial cell sources or may be chemically synthesized, but are preferably produced by recombinant

procedures involving host cells of the invention. Virulence gene products of the invention may be full length polypeptides, biologically active fragments, or variants thereof which retain specific biological or immunological activity. Variants may comprise virulence polypeptide analogs wherein one or more of the specified (*i.e.*, naturally encoded) amino acids is deleted or replaced or wherein one or more non-specified amino acids are added: (1) without loss of one or more of the biological activities or immunological characteristics specific for the virulence gene product; or (2) with specific disablement of a particular biological activity of the virulence gene product. Deletion variants contemplated also include fragments lacking portions of the polypeptide not essential for biological activity, and insertion variants include fusion polypeptides in which the wild-type polypeptide or fragment thereof have been fused to another polypeptide.

Variant virulence polypeptides include those wherein conservative substitutions have been introduced by modification of polynucleotides encoding polypeptides of the invention. Conservative substitutions are recognized in the art to classify amino acids according to their related physical properties and can be defined as set out in Table A (from WO 97/09433, page 10, published March 13, 1997 (PCT/GB96/02197, filed 9/6/96). Alternatively, conservative amino acids can be grouped as defined in Lehninger, [Biochemistry, Second Edition; Worth Publishers, Inc. NY:NY (1975), pp.71-77] as set out in Table B.

Table B
Conservative Substitutions II

SIDE CHAIN CHARACTERISTIC	AMINO ACID
Non-polar (hydrophobic)	
A. Aliphatic:	A L I V P
B. Aromatic:	F W
C. Sulfur-containing:	M
D. Borderline:	G
Uncharged-polar	
A. Hydroxyl:	S T Y
B. Amides:	N Q
C. Sulfhydryl:	C
D. Borderline:	G
Positively Charged (Basic):	K R H
Negatively Charged (Acidic):	
DE	

Variant virulence products of the invention include mature virulence gene products, *i.e.*, wherein leader or signal sequences are removed, having additional amino terminal residues. Virulence gene products having an additional methionine residue at position -1 are contemplated, as are virulence products having additional methionine and lysine residues at positions -2 and -1. Variants of these types are particularly useful for recombinant protein production in bacterial cell types. Variants of the invention also nclude gene products wherein amino terminal sequences derived from other proteins have been introduced, as well as variants comprising amino terminal sequences that are not found in naturally occurring proteins.

The invention also embraces variant polypeptides having additional amino acid residues which result from use of specific expression systems. For example, use of commercially available vectors that express a desired polypeptide as a fusion protein with glutathione-S-transferase (GST) provide the desired polypeptide having an additional glycine residue at position -1 following cleavage of the GST component from the desired polypeptide. Variants which result from expression using other vector systems are also contemplated.

Also comprehended by the present invention are antibodies (*e.g.*, monoclonal and polyclonal antibodies, single chain antibodies, chimeric antibodies,

humanized, human, and CDR-grafted antibodies, including compounds which include CDR sequences which specifically recognize a polypeptide of the invention) and other binding proteins specific for virulence gene products or fragments thereof. The term "specific for" indicates that the variable regions of the antibodies of the invention recognize and bind a virulence polypeptide exclusively (*i.e.*, are able to distinguish a single virulence polypeptides from related virulence polypeptides despite sequence identity, homology, or similarity found in the family of polypeptides), but may also interact with other proteins (for example, *S. aureus* protein A or other antibodies in ELISA techniques) through interactions with sequences outside the variable region of the antibodies, and in particular, in the constant region of the molecule. Screening assays to determine binding specificity of an antibody of the invention are well known and routinely practiced in the art. For a comprehensive discussion of such assays, see Harlow *et al.* (Eds), Antibodies A Laboratory Manual; Cold Spring Harbor Laboratory; Cold Spring Harbor , NY (1988), Chapter 6. Antibodies that recognize and bind fragments of the virulence polypeptides of the invention are also contemplated, provided that the antibodies are first and foremost specific for, as defined above, a virulence polypeptide of the invention from which the fragment was derived.

The DNA and amino acid sequence information provided by the present invention also makes possible the systematic analysis of the structure and function of the virulence genes and their encoded gene products. Knowledge of a polynucleotide encoding a virulence gene product of the invention also makes available anti-sense polynucleotides which recognize and hybridize to polynucleotides encoding a virulence polypeptide of the invention. Full length and fragment anti-sense polynucleotides are provided. The worker of ordinary skill will appreciate that fragment anti-sense molecules of the invention include (i) those which specifically recognize and hybridize to a specific RNA (as determined by sequence comparison of DNA encoding a virulence polypeptide of the invention to DNA encoding other known molecules) as well as (ii) those which recognize and hybridize to RNA encoding variants of the family of virulence proteins. Antisense polynucleotides that hybridize to RNA encoding other members of the virulence family of proteins are also identifiable through sequence comparison to identify characteristic, or signature, sequences for the family of molecules.

The invention further contemplates methods to modulate gene expression through use of ribozymes. For a review, see Gibson and Shillitoe, *Mol. Biotech. 7*:125-137 (1997). Ribozyme technology can be utilized to inhibit translation of mRNA in a sequence specific manner through (i) the hybridization of a complementary RNA to a target mRNA and (ii) cleavage of the hybridized mRNA through nuclease activity inherent to the complementary strand. Ribozymes can be identified by empirical methods but more preferably are specifically designed based on accessible sites on the target mRNA [Bramlage, *et al., Trends in Biotech 16*:434-438 (1998)]. Delivery of ribozymes to target cells can be accomplished using either exogenous or endogenous delivery techniques well known and routinely practiced in the art. Exogenous delivery methods can include use of targeting liposomes or direct local injection. Endogenous methods include use of viral vectors and non-viral plasmids.

Ribozymes can specifically modulate expression of virulence genes when designed to be complementary to regions unique to a polynucleotide encoding a virulence gene product. "Specifically modulate" therefore is intended to mean that ribozymes of the invention recognizes only a single polynucleotide. Similarly, ribozymes can be designed to modulate expression of all or some of a family of proteins. Ribozymes of this type are designed to recognize polynucleotide sequences conserved in all or some of the polynucleotides which encode the family of proteins.

The invention further embraces methods to modulate transcription of a virulence gene of the invention through use of oligonucleotide-directed triplet helix formation. For a review, see Lavrovsky, *et al., Biochem. Mol. Med. 62*:11-22 (1997). Triplet helix formation is accomplished using sequence specific oligonucleotides which hybridize to double stranded DNA in the major groove as defined in the Watson-Crick model. Hybridization of a sequence specific oligonucleotide can thereafter modulate activity of DNA-binding proteins, including, for example, transcription factors and polymerases. Preferred target sequences for hybridization include transcriptional regulatory regions that modulate virulence gene product expression. Oligonucleotides which are capable of triplet helix formation are also useful for site-specific covalent modification of target DNA sequences. Oligonucleotides useful for covalent

modification are coupled to various DNA damaging agents as described in Lavrovsky, *et al.* [*supra*].

The identification of *P. multocida* and *A. pleuropneumoniae* virulence genes renders the genes and gene products useful in methods for identifying anti-bacterial agents. Such methods include assaying potential agents for the ability to interfere with expression of virulence gene products represented by the DNA sequences set forth in any one of SEQ ID NOS: 1, 3, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 29, 31, 33, 37, 39, 41, 51, 53, 55, 57, 58, 60, 68, 70, 72, 74, 76, 78, 80, 82, 84, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, and 120, 122, 124, 126, 128, 130, 132, 134, 135, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 163, and 164, and species homologs thereof (*i.e.*, the genes represented by DNA sequences of SEQ ID NOS: 1, 3, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 29, 31, 33, 37, 39, 41, 51, 53, 55, 57, 58, 60, 68, 70, 72, 74, 76, 78, 80, 82, 84, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, and 120, 122, 124, 126, 128, 130, 132, 134, 135, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 163, and 164, encode the virulence gene product, or the DNA sequences of SEQ ID NOS: 1, 3, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 29, 31, 33, 37, 39, 41, 51, 53, 55, 57, 58, 60, 68, 70, 72, 74, 76, 78, 80, 82, 84, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, and 120, 122, 124, 126, 128, 130, 132, 134, 135, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 163, and 164, are adjacent the gene encoding the virulence gene product, or are involved in regulation of expression of the virulence gene product), or assaying potential agents for the ability to interfere with the function of a bacterial gene product encoded in whole or in part by a DNA sequence set forth in any one of SEQ ID NOs: 1, 3, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 29, 31, 33, 37, 39, 41, 51, 53, 55, 57, 58, 60, 68, 70, 72, 74, 76, 78, 80, 82, 84, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, and 120, 122, 124, 126, 128, 130, 132, 134, 135, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 163, and 164, species homologs thereof, or the complementary strand thereof, followed by identifying agents that are positive in such assays. Polynucleotides and polypeptides useful in these assays include not only the genes and encoded polypeptides as disclosed herein, but also variants thereof that have substantially the same activity as the wild-type genes and polypeptides.

The virulence gene products produced by the methods described above are used in high throughput assays to screen for inhibitory agents. The sources for potential agents to be screened are chemical compound libraries, fermentation media of *Streptomycetes,* other bacteria and fungi, and cell extracts of plants and other vegetations. For proteins with known enzymatic activity, assays are established based on the activity, and a large number of potential agents are screened for ability to inhibit the activity. For proteins that interact with another protein or nucleic acid, binding assays are established to measure such interaction directly, and the potential agents are screened for ability to inhibit the binding interaction.

The use of different assays known in the art is contemplated according to this aspect of the invention. When the function of the virulence gene product is known or predicted by sequence similarity to a known gene product, potential inhibitors can be screened in enzymatic or other types of biological and/or biochemical assays keyed to the function and/or properties of the gene product. When the virulence gene product is known or predicted by sequence similarity to a known gene product to interact with another protein or nucleic acid, inhibitors of the interaction can be screened directly in binding assays. The invention contemplates a multitude of assays to screen and identify inhibitors of binding by the virulence gene product. In one example, the virulence gene product is immobilized and interaction with a binding partner is assessed in the presence and absence of a putative inhibitor compound. In another example, interaction between the virulence gene product and its binding partner is assessed in a solution assay, both in the presence and absence of a putative inhibitor compound. In both assays, an inhibitor is identified as a compound that decreases binding between the virulence gene product and its binding partner. Other assays are also contemplated in those instances wherein the virulence gene product binding partner is a protein. For example, variations of the di-hybrid assay are contemplated wherein an inhibitor of protein/protein interactions is identified by detection of a positive signal in a transformed or transfected host cell as described in PCT publication number WO 95/20652, published August 3, 1995.

Candidate inhibitors contemplated by the invention include compounds selected from libraries of potential inhibitors. There are a number of different libraries used for the identification of small molecule modulators, including: (1) chemical

libraries, (2) natural product libraries, and (3) combinatorial libraries comprised of random peptides, oligonucleotides or organic molecules. Chemical libraries consist of structural analogs of known compounds or compounds that are identified as "hits" or "leads" via natural product screening. Natural product libraries are collections of microorganisms, animals, plants, or marine organisms which are used to create mixtures for screening by: (1) fermentation and extraction of broths from soil, plant or marine microorganisms or (2) extraction of plants or marine organisms. Natural product libraries include polyketides, non-ribosomal peptides, and variants (non-naturally occurring) thereof. For a review, see *Science 282*:63-68 (1998). Combinatorial libraries are composed of large numbers of peptides, oligonucleotides, or organic compounds as a mixture. They are relatively easy to prepare by traditional automated synthesis methods, PCR, cloning, or proprietary synthetic methods. Of particular interest are peptide and oligonucleotide combinatorial libraries. Still other libraries of interest include peptide, protein, peptidomimetic, multiparallel synthetic collection, recombinatorial, and polypeptide libraries. For a review of combinatorial chemistry and libraries created therefrom, see Myers, *Curr. Opin. Biotechnol. 8*:701-707 (1997). Identification of modulators through use of the various libraries described herein permits modification of the candidate "hit" (or "lead") to optimize the capacity of the "hit" to modulate activity.

Still other candidate inhibitors contemplated by the invention can be designed and include soluble forms of binding partners, as well as binding partners as chimeric, or fusion, proteins. Binding partners as used herein broadly encompasses antibodies, antibody fragments, and modified compounds comprising antibody domains that are immunospecific for the expression product of the identified virulence gene.

Other assays may be used when a binding partner (*i.e.,* ligand) for the virulence gene product is not known, including assays that identify binding partners of the target protein through measuring direct binding of test binding partner to the target protein, and assays that identify binding partners of target proteins through affinity ultrafiltration with ion spray mass spectroscopy/HPLC methods or other physical and analytical methods. Alternatively, such binding interactions are evaluated indirectly using the yeast two-hybrid system described in Fields and Song, *Nature, 340*:245-246

(1989), and Fields and Sternglanz, *Trends in Genetics, 10*:286-292 (1994), both of which are incorporated herein by reference. The two-hybrid system is a genetic assay for detecting interactions between two proteins or polypeptides. It can be used to identify proteins that bind to a known protein of interest, or to delineate domains or residues critical for an interaction. Variations on this methodology have been developed to clone genes that encode DNA-binding proteins, to identify peptides that bind to a protein, and to screen for drugs. The two-hybrid system exploits the ability of a pair of interacting proteins to bring a transcription activation domain into close proximity with a DNA-binding domain that binds to an upstream activation sequence (UAS) of a reporter gene, and is generally performed in yeast. The assay requires the construction of two hybrid genes encoding (1) a DNA-binding domain that is fused to a first protein and (2) an activation domain fused to a second protein. The DNA-binding domain targets the first hybrid protein to the UAS of the reporter gene; however, because most proteins lack an activation domain, this DNA-binding hybrid protein does not activate transcription of the reporter gene. The second hybrid protein, which contains the activation domain, cannot by itself activate expression of the reporter gene because it does not bind the UAS. However, when both hybrid proteins are present, the noncovalent interaction of the first and second proteins tethers the activation domain to the UAS, activating transcription of the reporter gene. When the virulence gene product (the first protein, for example) is already known to interact with another protein or nucleic acid, this assay can be used to detect agents that interfere with the binding interaction. Expression of the reporter gene is monitored as different test agents are added to the system; the presence of an inhibitory agent results in lack of a reporter signal.

When the function of the virulence gene product is unknown and no ligands are known to bind the gene product, the yeast two-hybrid assay can also be used to identify proteins that bind to the gene product. In an assay to identify proteins that bind to the first protein (the target protein), a large number of hybrid genes each encoding different second proteins are produced and screened in the assay. Typically, the second protein is encoded by a pool of plasmids in which total cDNA or genomic DNA is ligated to the activation domain. This system is applicable to a wide variety of proteins, and it is not even necessary to know the identity or function of the second binding protein. The

system is highly sensitive and can detect interactions not revealed by other methods; even transient interactions may trigger transcription to produce a stable mRNA that can be repeatedly translated to yield the reporter protein.

Other assays may be used to search for agents that bind to the target protein. One such screening method to identify direct binding of test ligands to a target protein is described in U.S. Patent No. 5,585,277, incorporated herein by reference. This method relies on the principle that proteins generally exist as a mixture of folded and unfolded states, and continually alternate between the two states. When a test ligand binds to the folded form of a target protein (i.e., when the test ligand is a ligand of the target protein), the target protein molecule bound by the ligand remains in its folded state. Thus, the folded target protein is present to a greater extent in the presence of a test ligand which binds the target protein, than in the absence of a ligand. Binding of the ligand to the target protein can be determined by any method which distinguishes between the folded and unfolded states of the target protein. The function of the target protein need not be known in order for this assay to be performed. Virtually any agent can be assessed by this method as a test ligand, including, but not limited to, metals, polypeptides, proteins, lipids, polysaccharides, polynucleotides and small organic molecules.

Another method for identifying ligands for a target protein is described in Wieboldt *et al.*, *Anal. Chem.*, *69*:1683-1691 (1997), incorporated herein by reference. This technique screens combinatorial libraries of 20-30 agents at a time in solution phase for binding to the target protein. Agents that bind to the target protein are separated from other library components by centrifugal ultrafiltration. The specifically selected molecules that are retained on the filter are subsequently liberated from the target protein and analyzed by HPLC and pneumatically assisted electrospray (ion spray) ionization mass spectroscopy. This procedure selects library components with the greatest affinity for the target protein, and is particularly useful for small molecule libraries.

The inhibitors/binders identified by the initial screens are evaluated for their effect on virulence in *in vivo* mouse models of *P. multocida* infections. Models of bacteremia, endocarditis, septic arthritis, soft tissue abscess, or pneumonia may be utilized. Models involving use of other animals are also comprehended by the invention.

For example, rabbits can be challenged with a wild type *P. multocida* strain before or after administration of varying amounts of a putative inhibitor/binder compound. Control animals, administered only saline instead of putative inhibitor/binder compound provide a standard by which deterioration of the test animal can be determined. Other animal models include those described in the Animal and Plant Health Inspection Sevice, USDA, January 1, 1994 Edition, §§113.69-113.70; Panciera and Corstvet, *Am. J. Vet. Res. 45*:2532-2537; Ames, *et al., Can. J. Comp. Med. 49*:395-400 (1984); and Mukkur, *Infection and Immunity 18*:583-585 (1977). Inhibitors/binders that interfere with bacterial virulence are can prevent the establishment of an infection or reverse the outcome of an infection once it is established.

Any adjuvant known in the art may be used in the vaccine composition, including oil-based adjuvants such as Freund's Complete Adjuvant and Freund's Incomplete Adjuvant, mycolate-based adjuvants (*e.g.,* trehalose dimycolate), bacterial lipopolysaccharide (LPS), peptidoglycans (*i.e.,* mureins, mucopeptides, or glycoproteins such as N-Opaca, muramyl dipeptide [MDP], or MDP analogs), proteoglycans (*e.g.,* extracted from *Klebsiella pneumoniae*), streptococcal preparations (*e.g.,* OK432), Biostim™ (*e.g.,* 01K2), the "Iscoms" of EP 109 942, EP 180 564 and EP 231 039, aluminum hydroxide, saponin, DEAE-dextran, neutral oils (such as miglyol), vegetable oils (such as arachis oil), liposomes, Pluronic® polyols, the Ribi adjuvant system (see, for example GB-A-2 189 141), or interleukins, particularly those that stimulate cell mediated immunity. An alternative adjuvant consisting of extracts of *Amycolata*, a bacterial genus in the order Actinomycetales, has been described in U.S. Patent No. 4,877,612. Additionally, proprietary adjuvant mixtures are commercially available. The adjuvant used will depend, in part, on the recipient organism. The amount of adjuvant to administer will depend on the type and size of animal. Optimal dosages may be readily determined by routine methods.

The vaccine compositions optionally may include vaccine-compatible pharmaceutically acceptable (*i.e.,* sterile and non-toxic) liquid, semisolid, or solid diluents that serve as pharmaceutical vehicles, excipients, or media. Any diluent known in the art may be used. Exemplary diluents include, but are not limited to, polyoxyethylene sorbitan monolaurate, magnesium stearate, methyl- and

propylhydroxybenzoate, talc, alginates, starches, lactose, sucrose, dextrose, sorbitol, mannitol, gum acacia, calcium phosphate, mineral oil, cocoa butter, and oil of theobroma.

The vaccine compositions can be packaged in forms convenient for delivery. The compositions can be enclosed within a capsule, caplet, sachet, cachet, gelatin, paper, or other container. These delivery forms are preferred when compatible with entry of the immunogenic composition into the recipient organism and, particularly, when the immunogenic composition is being delivered in unit dose form. The dosage units can be packaged, *e.g.*, in tablets, capsules, suppositories or cachets.

The vaccine compositions may be introduced into the subject to be immunized by any conventional method including, *e.g.*, by intravenous, intradermal, intramuscular, intramammary, intraperitoneal, or subcutaneous injection; by oral, sublingual, nasal, anal, or vaginal, delivery. The treatment may consist of a single dose or a plurality of doses over a period of time.

The invention also comprehends use of an attenuated bacterial strain of the invention for manufacture of a vaccine medicament to prevent or alleviate bacterial infection and/or symptoms associated therewith. The invention also provides use of inhibitors of the invention for manufacture of a medicament to prevent or alleviate bacterial infection and/or symptoms associated therewith.

The present invention is illustrated by the following examples. Example 1 describes constructions of *P. multocida* mutants. Example 2 relates to screening for *P. multocida* mutants. Example 3 addresses methods to determine virulence of the *P. multocida* mutants. Example 4 describes cloning of *P. multocida* virulence genes. Example 5 addresses identification of genes in other species related to *P. multocida* virulence genes. Example 6 describes construction of *A. pleuropneumoniae* mutants. Example 7 addresses screening for attenuated *A. pleuropneumoniae* mutants. Example 8 relates to identification of *A. pleuropneumoniae* virulence genes. Example 9 describes competition challenge of *A. pleuropneumoniae* mutants and wild type bacteria. Example 10 characterizes *A. pleuropneumoniae* genes identified. Example 11 addresses efficacy of *A. pleuropneumoniae* mutant to protect against wild type bacterial challenge.

Example 1
Construction of a Library of Tagged-Transposon *P. multocida* Mutants

A library of tagged-transposon mutants was constructed in parental vector pLOF/Km [Herrero, *et al., J Bacteriol. 172*:6557-67 (1990)] which has previously been demonstrated to be functional and random in *P. multocida* [Lee, *et al., Vet Microbiol. 50*:143-8 (1996)]. Plasmid pLOF/Km was constructed as a modification of suicide vector pGP704 and included a transposase gene under control of the *Tac* promoter as well as the mini-Tn10 transposable element encoding kanamycin resistance. Plasmid pTEF-1 was constructed as described below by modifying pLOF/Km to accept sequence tags which contained a semi-random $[NK]_{35}$ sequence.

Plasmid pLOF/Km was first modified to eliminate the unique *Kpn*I restriction site in the multiple cloning region and then to introduce a new *Kpn*I site in the mini-Tn10 region. The plasmid was digested with *Kpn*I and the resulting overhanging ends were filled in with Klenow polymerase according to manufacturer's suggested protocol. Restriction digests and ligations described herein were performed according to manufacturer's suggested protocols (Gibco BRL, Gaithersburg, MD and Boehringer Mannheim, Indianapolis, IN). The blunt end product was self-ligated to produce a plasmid designated pLOF/Km--*Kpn*I which was transformed into *E.coli* DH5α:λpir for amplification. *E.coli* DH5α: (λpir φ80dlacZΔM15, recA1, endA1, gyrA96, thi-1, hsdR17(r_k^-, m_k, supE44, relA1, deoR, Δ(lacZYA-argF)U169, was propagated at 37°C in Luria-Bertani (LB) medium. Plasmids were prepared using QIAGEN SpinPreps from QIAGEN Inc. (Santa Clarita, CA) and digested with *Sfi*I which cuts at a unique site within the mini-Tn10 transposable element. A *Sfi*I-*Kpn*I-*Sfi*I adaptor was prepared by annealing oligonucleotides TEF1 (SEQ ID NO: 86) and TEF3 (SEQ ID NO: 87) and the resulting double-stranded adapter was ligated into the *Sfi*I site to create plasmid pTEF-1. Oligonucleotides TEF1 and TEF3 (as well as all other oligonucleotides described herein) were synthesized by Genosys Biotechnologies (The Woodlands, TX).

TEF1 5′-AGGCCGGTACCGGCCGCCT SEQ ID NO: 86

TEF3 5′-CGGCCGGTACCGGCCTAGG SEQ ID NO: 87

Unique sequence tags for insertion into the *Kpn*I site of pTEF-1 were prepared as follows. PCR was carried out to generate double stranded DNA tags using a GeneAmp XL PCR Kit (PE Applied Biosystems, Foster City, CA) under conditions including 250 μM each dNTP, 1.5 mM $Mg(OAc)_2$, 100 pmol each primer TEF14 (SEQ ID NO: 88) and TEF15 (SEQ ID NO: 89), 1 ng TEF26 (SEQ ID NO: 90) as template DNA and 2.5 units recombinant *Tth* DNA Polymerase XL.

TEF14	5′-CATGGTACCCATTCTAAC	SEQ ID NO: 88
TEF15	5′-CTAGGTACCTACAACCTC	SEQ ID NO: 89
TEF26		SEQ ID NO: 90

5′-CTAGGTACCTACAACCTCAAGCTT-$[NK]_{35}$-
AAGCTTGGTTAGAATGGGTACCATG

Reaction conditions included an initial incubation at 95°C for one minute, followed by thirty cycles of 30 seconds at 95°C, 45 seconds at 45°C, and 15 seconds at 72°C, followed by a final incubation at 72°C for two minutes. The PCR products were digested with *Kpn*I and purified using a QIAGEN Nucleotide Removal Kit (QIAGEN, Inc., Chatsworth, GA) according to the manufacturer's suggested protocol. The unique tag sequences were ligated into the mini-Tn10 element of linearized pTEF-1, previously digested with *Kpn*I and dephosphorylated with calf intestinal alkaline phosphatase (Boehringer Mannheim) using standard procedures. The resulting plasmid library was transformed into *E.coli* DH5α:λpir. Colony blot analysis was performed according to the DIG User's Guide (Boehringer-Mannheim) with hybridization and detection performed as follows.

Hybridizations were essentially performed according to the Genius Non-Radioactive User's Guide (Boehringer Mannheim Biochemicals), the product sheet for the DIG-PCR labeling kit (Boehringer Mannheim Biochemicals), and the product sheet for CSPD (Boehringer Mannheim Biochemicals). For preparation of probes, a 100 μl primary PCR reaction was set up using Amplitaq PCR buffer (PE Applied Biosystems),

200 μM dNTPs, 140 pmol each of primers TEF5 (SEQ ID NO: 91) and TEF6 (SEQ ID NO: 92), 2 mM $MgCl_2$, 2.5 units Amplitaq (PE Applied Biosystems) and 1 ng of plasmid DNA.

TEF5	5′-TACCTACAACCTCAAGCT	SEQ ID NO: 91
TEF6	5′-TACCCATTCTAACCAAGC	SEQ ID NO: 92

Cycle conditions included an initial incubation at 95°C for two minutes, followed by 35 cycles of 95°C for 30 seconds, 50°C for 45 seconds, 72°C for 15 seconds and a final incubation at 72°C for three minutes. The amplification products were separated using electrophoresis on a 2% - 3:1 NuSieve GTG (FMC BioProducts, Rockland, ME, USA):Agarose gel and the 109 bp product was excised and purified. Gel extractions were carried out using a QIAGEN Gel Extraction kit (QIAGEN). Approximately 15 ng of the primary product was labeled in a 50 μl PCR reaction using the DIG PCR Kit, 50 pmol each of primers TEF24 and TEF25, and a 1:1 mix of DIG Probe Synthesis Mix with 2 mM dNTP stock solution.

TEF24	5′-TACCTACAACCTCAAGCTT	SEQ ID NO: 93
TEF25	5′-TACCCATTCTAACCAAGCTT	SEQ ID NO: 94

PCR conditions included an initial incubation at 95°C for four minutes, followed by 25 cycles of 95°C for 30 seconds, 50°C for 45 seconds, 72°C for 15 seconds and a final incubation at 72°C for three minutes. The labeled PCR product was digested with *Hin*dIII in a total reaction volume of 90 μl and purified from the constant primer arms using a 2% - 3:1 NuSieve GTG (FMC BioProducts):Agarose gel. The region containing the labeled variable tag was excised and the entire gel slice was dissolved and denatured in 10 ml of DIG EasyHyb at 95°C for ten minutes.

Dot blots were prepared using a Hybond®-N^+ membrane (Amersham-Pharmacia Biotech). Target DNA for each tag was prepared in 96 well plates using

approximately 30 ng of PCR product. An equal volume of 0.1 N NaOH was added to denature the sample and each sample was applied to the membrane with minimal vacuum using a Minifold I™ Dot-Blot Apparatus from Schleicher and Schuell (Keene, NH, USA). Each well was washed with 150 µl of Neutralization Solution (0.5 M Tris /3 M NaCl, pH 7.5) and 150 µl of 2X SSC. Membranes were UV-crosslinked in a Stratalinker (Stratagene, La Jolla, CA, USA) and prehybridized for one hour in 20 mls DIG EasyHyb Buffer at 42°C. The denatured probe was added and hybridization carried out overnight at 42°C. The membrane was washed two times in 2X SSC containing 0.1% SDS for five minutes each wash. Two high stringency washes were performed in 50 ml of pre-warmed 0.1X SSC buffer containing 0.1% SDS at 68°C for 15 minutes before proceeding with standard Genius Detection protocols (Genius Manual).

It is desirable to use a non-radioactive detection system for safety, lower cost, ease of use, and reduction of hazardous materials. In initial experiments using similar procedures previously described [Mei, *et al., Mol Microbiol. 26*:399-407 (1997)], unacceptable background levels of hybridization were obtained in negative controls. In order to decrease background, tag length was increased by 30 bp to a total of 70, amplification primers were lengthened to include all sequence flanking the variable region, a lower concentration of dig-dUTP was used, and the conserved sequences flanking the sequence tag region were removed by gel purification. Most significantly, PCR was used to generate $[NK]_{35}$ sequence tags as the target DNA in dot blots rather than the entire plasmids containing the tagged transposons after detecting background hybridization from the transposon itself. Using these modifications background was eliminated making chemiluminescent/non-radioactive screening more effective.

Approximately four hundred different transformants resulting from the ligation of pTEF-1 with the PCR generated sequence tags were screened by colony blot and the 96 strongest hybridizing colonies were assembled into microtiter plates for further use. Even though the likelihood of duplicated tags was very low, half of the plate of master tags was probed against the other to confirm that no tags were duplicated. The plasmids containing these tags were purified and transformed into *E.coli* S17-1:λpir (pir, *recA*, *thi*, *pro*, *hsd*, (r-m+), RP4-2, (Tc::Mu), (Km::Tn7), [TmpR], [SmR]), and the transformed bacteria propagated at 37°C in Luria-Bertani (LB) medium. Each of the 96

E.coli S17-1:λpir transformants containing the tagged plasmid pTEF-1 was used in conjugative matings to generate transposon mutants of *P. multocida*. *P. multocida* strain TF5 is a spontaneous nalidixic acid resistant mutant derived from UC6731, a bovine clinical isolate. *P. multocida* strains were grown on brain heart infusion (BHI) media (Difco Laboratories, Detroit, MI, USA) at 37°C and in 5% CO_2 when grown on plates. Matings were set up by growing each *E.coli* S17-1:λpir /pTEF1:$[NK]_{35}$ clone and the TF5 strain to late log phase. Fifty μl of culture for each tagged-pTEF-1 clone was mixed with 200 μl of the TF5 culture and 50 μl of each mating mixture was spotted onto 0.22 TM filters previously placed on BHI plates containing 100 mM IPTG and 10 mM $MgSO_4$. Following overnight incubation at 37°C with 5% CO_2, mating mixtures were washed off of each filter into 3 ml of PBS and 25 μl of each was plated onto $BHIN^{50}K^{100}$ plates. Following selective overnight growth, colonies were assembled into microtiter plates by toothpick transfer into 200 μl $BHIN^{50}K^{50}$ making sure that each well in a microtiter plate always contained a transposon mutant with the same sequence tag. Following overnight growth, 50 μl of 75% glycerol was added to each well and plates were stored frozen at -80°C.

Nineteen pools were assembled by transferring the transposon mutants to microtiter plates making sure that each well contained a transposon mutant with the appropriate tag for that well. In other words, a specific well in each microtiter plate always contained a transposon mutant with the same sequence tag even though the location of the transposon within those mutants may be different.

Example 2
Murine Screening for Attenuated *P. multocida* Mutants

Nineteen pools of *Pasteurella multocida* transposon mutants were screened using a murine model of septicemia. Frozen plates of pooled *P. multocida* transposon mutants were removed from -80°C storage and subcultured by transferring 10 μl from each well to a new 96 well round bottom plate (Corning Costar, Cambridge, MA, USA) containing 200 μl of brain heart infusion (DIFCO) with 50 μg/ml nalidixic acid (Sigma) and 50 μg/ml kanamycin (Sigma) ($BHIN^{50}K^{50}$). Plates were incubated without shaking overnight at 37°C in 5% CO_2. Overnight plates were subcultured by transferring 10 μl from each well to a new flat bottomed 96-well plate (Corning Costar)


containing 100 µl of BHI per well and incubating at 37°C with shaking at approximately 150 rpm. The OD_{540} was monitored using a micro-titer plate reader. At an OD_{540} of approximately 0.2 to 0.25, each plate was pooled to form the "input pool" by combining 100 µl from each of the wells of the micro-titer plate. The culture was diluted appropriately in BHI to doses of approximately 10^4, 10^5, 10^6 CFU/ml and 0.2 ml of each dilution was used to infect female 14-16 g BALB/c mice by intraperitoneal administration. At two days post-infection, one or two surviving mice were euthanized and the spleens harvested. The entire spleen was homogenized in 1.0 ml sterile 0.9 % saline. Dilutions of the homogenate from 10^{-2} to 10^{-5} were prepared and plated onto $BHIN^{50}K^{50}$ plates. Following overnight growth, at least 20,000 colonies were pooled in 10 mls BHI broth to form the "recovered pool" and 0.5 ml of the recovered pool was centrifuged at 3,500 X g and the pellet used to prepare genomic DNA according to a previously described protocol [Wilson, *In* F. M. Ausubel, *et al.*,(ed.), Current Protocols in Molecular Biology, vol. 1. John Wiley and Sons, New York, p. 2.4.1-2.4.5. (1997)].

Initial experiments with virulent wild-type *P. multocida* indicated that organisms could be recovered from the spleen, lungs, kidneys, and liver indicating a truly septicemic model of infection. Dot blots for both the "input" and "recovered" pools were performed as described in Example 1 and evaluated both by visual inspection and by semi-quantitative analysis. Hybridization was carried out as described in Example 1 except that 5 µg of genomic DNA from input and recovered pools was used as template. Semi-quantitative analysis indicates whether a significant reduction in a single clone has occurred. If a mutant is unable to survive within the host, then the recovered signal should be very low compared to the input signal yielding a high input/recovered ratio. Most mutants will grow as well *in vivo* as *in vitro* and therefore a ratio of their signals should be approximately equal to 1. Clones selected by quantitative analysis as being highly reduced in the recovered pool were selected for further study. Additional clones with questionable input/recovered ratios were also selected after visually evaluating films made from the dot blots.

Example 3
Determination of Virulence for *P. multocida* Candidate Mutants

Each potential mutant which exhibited reduced recovery from splenic tissue was isolated from the original pool plate and used individually in a challenge experiment to verify and roughly estimate the attenuation caused by the transposon mutation. Individual candidate mutants from *in vivo* screens were grown on Sheep Blood Agar plates overnight in 5% CO_2 at 37°C. Approximately six colonies of each mutant were inoculated into BHI broth and allowed to grow for six hours. Dilutions were prepared and five mice each were infected as described above with 10^2, 10^3, 10^4 and 10^5 CFU each. Attenuation was determined by comparing mortality after six days relative to the wild type. Surviving mice were presumed to be protected and then challenged with a dose of wild type *P. multocida* at a concentration approximately 200-fold greater than the LD_{50} for the wild type strain. Survival rate was then determined for each challenged group of mice.

Results indicated that 62 of 120 potential transposon mutants were attenuated, having an approximate LD_{50} of at least 10 fold higher than the wild type strain. The clones and their approximate LD_{50} values are listed in Table 1. A control experiment with the wild type strain was run in parallel with each set of challenges and in all cases mortality in wild type-challenged groups was 100%.

In addition to LD_{50} values, Table 1 also provides data from vaccination and challenge experiments. Briefly, groups of mice (n = 5 to 10) were vaccinated by intraperitoneal injection with the individual *P. multocida* strains shown in Table 1 at a dose that was approximately 200 times greater than the LD_{50} of the virulent, wild type strain. Animals were observed for 28 days after which mortality figures were calculated.

Table 1

P. multocida Virulence Genes

Nucleotide SEQ ID NO:	Representative Isolate	PossibleGene Function	Vaccination # survivors/total	Challenge # survivors/total	LD_{50}
--	wild type	-	0/10	-	<10
23	PM1B1	guaB	10/10, 10/10, 10/10	9/10, 9/10	4.3 x 106
11	PM1D1	dsbB	10/10, 5/10	10/10, 5/5	8.4 x 104
3	PM1BD7	atpG	5/5, 10/10	10/10	>3 x 105
74	PM1BE11	yhcJ (HI0145)	10/10	5/10	>2 x 105
70	PM1BF6	yabK (HI1020)	3/5, 8/10	9/9	>2 x 105
19	PM2G8	fhaC	4/5, 9/10	9/9	>4 x 105

Nucleotide SEQ ID NO:	Representative Isolate	PossibleGene Function	Vaccination # survivors/total	Challenge # survivors/total	LD_{50}
76	PM3C9	yiaO (HI0146)	3/5		>6 x 105
118	PM3G11	UnkO	4/5, 10/10	10/10	>3 x 105
31	PM7B4	iroA (UnkB)	0/5		
17	PM4C6	fhaB (fhaB2)	2/5, 10/10, 9/10	10/10, 9/9	>3 x 106
9	PM4G10-T9	dnaA	4/5		>5 x 105
1	PM4D5-T5	atpB	5/5		>4 x 105
53	PM4D5-T1	UnkC2	5/5		>4 x 105
15	PM4F2	fhaB (fhaB1)	3/5, 6/10, 10/10	6/6, 10/10	>3 x 105
41	PM5F7	mreB	4/5		1 x 103
7	PM5E2	devB	0/5, 3/10	2/3	?
68	PM6H5-T1	xylA	5/5		>3 x 105
78	PM6H8	yigF (HI0719)	5/5, 9/10	9/9	>3 x 105
108	PM7D12	pnp	5/5, 9/10	9/9	
51	PM8C1R1-T2	UnkC1	5/5		~6 x 105
37	PM8C1-T3	mglB	5/5		~6 x 105
58	PM8C1R1-T6	UnkD1	5/5		~6 x 105
45	PM10H7	purF (HI1207)	3/5, 8/10, 8/10	8/8, 8/8	>3 x 105
25	PM10H10-T2	HI1501	5/5		>1 x 104
72	PM11G8-T2	ygiK	5/5		>2.4 x 103
21	PM11G8-T4	greA	5/5		>2.4 x 103
84	PM12H6	yyam (HI0687)	3/5, 0/10		~2.2 x 103
33	PM15G8-T2	kdtB	5/5		>1.2 x 105
116	PM15G8-T1	UnkK	5/5		>1.2 x 105
104	PM16G11-T1	hmbR	3/5		>1.9 x 105
29	PM16G11-T2	hxuC	3/5		>1.9 x 105
35	PM16H8	lgtC	5/5, 10/10	10/10	>2.4 x 105
80	PM16H3	yleA (HI0019)	5/5, 10/10		> 2.0 x 105
49	PM17H6-T1	sopE	4/5		~6 x 105
120	PM17H6	UnkP	4/5		~6 x 105
5	PM18F5-T8	cap5E	5/5		>2.4 x 105
82	PM18F5-T10	yojB (HI0345)	5/5		>2.4 x 105
13	PM19A1	exbB	5/5, 10/10	10/10	>1.2 x 105
112	PM19D4	rci	5/5, 8/10	8/8	~1.6 x 105
39	PM20A12	mioC (HI0669)	3/5, 8/10	8/8	~2 x 104
60	PM20C2	UnkD2	5/5, 10/10	10/10	>8.2 x 106

Example 4
Cloning and Identification of Genes Required for *P. multocida* Virulence

Each transposon mutant which was verified to be attenuated was analyzed further to determine the identity of the disrupted open reading frame. DNA from each mutant was amplified, purified, and digested with restriction enzymes that were known not to cut within the transposon and generally produced 4-8 kb fragments that hybridized with the transposon. Using selection for kanamycin resistance encoded by the transposon, at least one fragment for each transposon mutant was cloned.

Southern hybridization with multiple restriction enzymes was performed for each attenuated mutant using a labeled 1.8 kb *Mlu*I fragment from pLOF/Km as a probe to identify a suitably sized fragment for cloning. The mini-Tn10 element and

flanking DNA from each mutant was cloned into pUC19 and the flanking sequence determined using internal primers TEF32 and TEF40, primer walking and in some cases universal pUC-19 primers.

TEF-32	GGCAGAGCATTACGCTGAC	SEQ ID NO: 95
TEF-40	GTACCGGCCAGGCGGCCACGCGTATTC	SEQ ID NO:96

Sequencing reactions were performed using the BigDye™ Dye Terminator Chemistry kit from PE Applied Biosystems (Foster City, CA) and run on an ABI Prism 377 DNA Sequencer. Double stranded sequence for putative interrupted open reading frames was obtained for each clone. Sequencer 3.0 software (Genecodes, Corp., Ann Arbor, MI) was used to assemble and analyze sequence data. GCG programs [Devereux, *et al.*, 1997. Wisconsin Package Version 9.0, 9.0 ed. Genetics Computer Group, Inc., Madison] were used to search for homologous sequences in currently available databases.

In 37% of the clones that were identified as being attenuated, there were multiple insertions of the mini-Tn10 transposable element. Each insertion including its flanking sequence was cloned individually into pGP704 and mated into the wild-type strain to produce new mutants of *P. multocida*, each carrying only one of the multiple original insertions. Individual mutants were retested individually to determine the insertion responsible for the attenuated phenotype. The nucleotide sequence of the disrupted, predicted open reading frame was determined by sequencing both strands, and the predicted amino acid sequence was used to search currently available databases for similar sequences. Sequences either matched known genes, unknown genes, and hypothetical open reading frames previously sequenced or did not match any previously identified sequence. For those genes having homology to previously identified sequences, potential functions were assigned as set out in Table 1.

Example 5
Identification of Related Genes in Other Species

In separate experiments, STM was also performed using *Actinobacillus pleuropneumoniae* (App). One of the App strains contained an insertion in a gene that was sequenced (SEQ ID NO: 97) and identified as a species homolog of the *P. multocida*

atpG gene. This result suggested the presence in other bacterial species of homologs to previously unknown *P. multocida* genes that can also be mutated to produce attenuated strains of the other bacterial species for use in vaccine compositions. In order to determine if homologs of other *P. multocida* genes exists in other bacterial species, Southern hybridization was performed on genomic DNA from other species using the *A. pleuropneumoniae atpG* gene as a probe.

Actinobacillus pleuropneumoniae, *Pasteurella haemolytica* (Ph), *P. multocida*, and *Haemophilus somnus* (Hs) genomic DNA was isolated using the CTAB method and digested with *Eco*RI and *Hin*dIII for two hours at 37°C. Digested DNA was separated on a 0.7% agarose gel at 40V in TAE buffer overnight. The gel was immersed sequentially in 0.1 M HCL for 30 minutes, twice in 0.5 M NaOH/1.5 M NaCl for 15 minutes each, and twice in 2.5 M NaCl/1 M Tris, pH 7.5. The DNA was transferred to nitrocellulose membranes (Amersham Hybond N^+) overnight using 20X SSC buffer (3 M NaCl/0.3 M sodium citrate). The DNA was crosslinked to the membrane using a UV Stratalinker on autocrosslink setting (120 millijoules). The membrane was prehybridized in 5X SSC/ 1% blocking solution/0.1% sodium lauroyl sarcosine/0.02% SDS at 50°C for approximately seven hours and hybridized overnight at 50°C in the same solution containing a PCR generated atgG probe.

The probe was prepared using primers DEL-1389 (SEQ ID NO: 98) and TEF-46 (SEQ ID NO: 99) in a with a GeneAmp XL PCR kit in a GeneAmp PCR System 2400. Template was genomic *A. pleuropneumoniae* DNA.

DEL-1389	TCTCCATTCCCTTGCTGCGGCAGGG	SEQ ID NO: 98
TEF-46	GGAATTACAGCCGGATCCGGG	SEQ ID NO: 99

The PCR was performed with an initial heating step at 94°C for five minutes, 30 cycles of denaturation t 94°C for 30 sec, annealing at 50°C for 30 sec, and elongation at 72°C for three minutes, and a final extension step at 72°C for five minutes. The amplification products were separated on an agarose gel, purified using a QIAquick gel purification kit (QIAGEN), and labeled using a DIG-High Primer kit (Boehringer Mannheim). The blot was removed from the hybridization solution and rinsed in 2X SSC and washed two times for five minutes each wash in the same buffer. The blot was then washed two

times for 15 minutes each in 0.5X SSC at 60°C. Homologous bands were visualized using a DIG Nucleic Acid Detection Kit (Boehringer Mannheim).

Single bands were detected in *Pasteurella haemolytica, Haemophilus somnus* and *A. pleuropneumoniae* using *Eco*RI digested DNA. Two bands were detected using *Eco*Ri digested DNA from *Pasteurella multocida.*

Example 6
Construction of a Library of Tagged-Transposon *P. multocida* Mutants

Transposon mutagenesis using pLOF/Km has previously been reported to be functional and random in *A. pleuropneumoniae* [Tascon, *et al., J Bacteriol. 175*:5717-22 (1993)]. To construct tagged transposon mutants of *A. pleuropneumoniae*, each of 96 *E. coli* S17-1:λpir transformants containing pre-selected tagged plasmids (pTEF-1:$[NK]_{35}$) was used in conjugative matings to generate transposon mutants of *A. pleuropneumoniae* strain AP225, a serotype 1 spontaneous nalidixic acid resistant mutant derived from an in vivo passaged ATCC 27088 strain. *A. pleuropneumoniae* strains were grown on Brain Heart Infusion (BHI) (Difco Laboratories, Detroit, MI) media with 10 μg/ml B-nicotinamide adenine dinucleotide (V^{10}), (Sigma, St. Louis, Missouri) at 37°C and in 5% CO_2 when grown on plates. *E.coli* S17-1:λpir (λpir, *recA*, *thi*, *pro*, *hsdR*(r_k-,m_k+), RP4-2, (Tc^R::Mu), (Km^R::Tn7), [Tmp^R], [Sm^R]) was propagated at 37°C in Luria-Bertani (LB) medium. Antibiotics when necessary were used at 100 μg/ml ampicillin (Sigma), 50 μg/ml nalidixic acid (N^{50})(Sigma), and 50 (K^{50}) or 100 (K^{100}) μg/ml of kanamycin (Sigma).

Matings were set up by growing each *E. coli* S17-1:λpir/pTEF1:$[NK]_{35}$ clone and the AP225 strain to late log phase. A 50 μl aliquot of culture for each tagged-pTEF-1 clone was mixed with 150 μl of the APP225 culture, and then 50 μl of each mating mixture was spotted onto 0.22 μM filters previously placed onto $BHIV^{10}$ plates containing 100 μM IPTG and 10 mM $MgSO_4$. Following overnight incubation at 37°C with 5% CO_2, mating mixtures were washed off of each filter into 2 ml of PBS and 200 μl of each was plated onto $BHIV^{10}N^{50}K^{100}$ plates. After selective overnight growth, colonies were assembled into microtiter plates by toothpick transfer into 200 μl $BHIV^{10}N^{50}K^{50}$ making sure that each well in a microtiter plate always contained a

transposon mutant with the same sequence tag. Following overnight growth, 50 µl of 75% glycerol was added to each well and plates were stored frozen at -80°C.

APP does not appear to have as much bias towards multiple insertions of the mini-Tn10 element as did P. multocida. Only approximately 3% of the mutants were determined to contain multiple insertions, which is in agreement with the 4% previously reported [Tascon, *et al., J Bacteriol. 175*:5717-22 (1993)]. A problem in APP consisted of identifying numerous mutants (discussed below) containing insertions into 23S RNA regions: 28 total mutants with insertions into 13 unique sites. This may indicate that 23S RNA contains preferential insertion sites and that the growth of APP is affected by these insertions enough to result in differential survival within the host. Southern blot analysis using an APP 23S RNA probe suggests that APP may contain only three ribosomal operons as compared to five in *H. influenzae* [Fleischmann, *et al., Science 269*:496-512 (1995)] and seven complete operons in *E. coli* [Blattner, *et al., Science 277*:1453-1474 (1997)]. This site preference and its effect on growth rate may be a significant barrier to "saturation mutagenesis" since a significant number of clones will contain insertions into these rRNAs and large volume screening will be necessary to obtain additional unique attenuating mutations.

Example 7
Porcine Screening for Attenuated *A. pleuropneumoniae* Mutants

Twenty pools of *A. pleuropneumoniae* transposon mutants, containing a total of approximately 800 mutants, were screened using a porcine intratracheal infection model. Each pool was screened in two separate animals.

Frozen plates of pooled *A. pleuropneumoniae* transposon mutants were removed from -80°C storage and subcultured by transferring 20 µl from each well to a new 96 well round bottom plate (Corning Costar, Cambridge, MA, USA) containing 180 µl of $BHIV^{10}N^{50}K^{50}$. Plates were incubated without shaking overnight at 37°C in 5% CO_2. Overnight plates were then subcultured by transferring 10 µl from each well to a new flat bottomed 96 well plate (Corning Costar) containing 100 µl of $BHIV^{10}$ per well and incubating at 37°C with shaking at 150 rpm. The OD_{562} was monitored using a microtiter plate reader. At an OD_{562} of approximately 0.2 to 0.25, each plate was pooled to form the "input pool" by combining 100 µl from each of the wells of the microtiter

plate. The culture was diluted appropriately in BHI to approximately 2 X 10^6 CFU/ml. For each diluted pool, 4.0 ml was used to infect 10-20 kg SPF pigs (Whiteshire-Hamroc, Albion, IN) by intratracheal administration using a tracheal tube. At approximately 20 hours post-infection, all surviving animals were euthanized and the lungs removed. Lavage was performed to recover surviving bacteria by infusing 150 mls of sterile PBS into the lungs, which were then massaged to distribute the fluid. The lavage fluid was recovered, and the process was repeated a second time. The lavage fluid was centrifuged at 450 x g for 10 minutes to separate out large debris. Supernatants were then centrifuged at 2,800 x g to pellet the bacteria. Pellets were resuspended in 5 mls BHI and plated in dilutions ranging from 10^{-2} to 10^{-5} onto BHIV^{10}N^{50}K^{50} plates. Following overnight growth, at least 100,000 colonies were pooled in 10 mls BHI broth to form the "recovered pools". A 0.7 ml portion of each recovered pool was used to prepare genomic DNA by the CTAB method [Wilson, *In* Ausubel, *et al.*, (eds.), Current Protocols in Molecular Biology, vol. 1. John Wiley and Sons, New York, p. 2.4.1-2.4.5 (1997)].

Recovery from the animals routinely was in the 10^8 CFU range from lung lavage.

Dot blots were performed and evaluated both by visual inspection and by semi-quantitative analysis as described previously. All hybridizations and detections were performed as described. Briefly, probes were prepared by a primary PCR amplification, followed by agarose gel purification of the desired product and secondary PCR amplification incorporating dig-dUTP. Oligonucleotides including TEF5, TEF6, TEF24, TEF25, TEF48 and TEF62, were synthesized by Genosys Biotechnologies (The Woodlands, TX). Primers TEF69, TEF65, and TEF66 were also used for inverse PCR reactions and sequencing.

TEF69	GACGTTTCCCGTTGAATATGGCTC	SEQ ID NO: 166
TEF65	GCCGGATCCGGGATCATATGACAAGA	SEQ ID NO: 167
TEF66	GACAAGATGTGTATCCACCTTAAC	SEQ ID NO: 168

The labeled PCR product was then digested with *Hin*dIII to separate the constant primer arms from the unique tag region. The region containing the labeled variable tag was excised and the entire gel slice was then dissolved and denatured in DIG

EasyHyb. Dot blots were prepared and detected using the standard CSPD detection protocol. Film exposures were made for visual evaluation, and luminescent counts per second (LCPS) were determined for each dot blot sample. The $LCPS_{input}$ / $LCPS_{recovered}$ ratio for each mutant was used to determine mutants likely to be attenuated.

Clones selected as being present in the input pool but highly reduced in the recovered pool were selected for further study. Additional clones with questionable input/recovered ratios were also selected after visually evaluating films made from the dot blots. A total of 110 clones were selected.

Example 8
Identification of *A. pleuropneumoniae* Virulence Genes

A partial flanking sequence was determined for each of the 110 mutants by inverse PCR and direct product sequencing. Inverse PCR was used to generate flanking DNA products for direct sequencing as described above. Sequencing reactions were performed using the BigDye™ Dye Terminator Chemistry kit from PE Applied Biosystems (Foster City, CA) and run on an ABI Prism 377 DNA Sequencer. Sequencher 3.0 software (Genecodes, Corp., Ann Arbor, MI) was used to assemble and analyze sequence data. GCG programs [Devereux and Haeberli, Wisconsin Package Version 9.0, 9.0 ed. Genetics Computer Group, Inc., Madison (1997)] were used to search for homologous sequences in currently available databases.

Table 2 shows the *A. pleuropneumoniae* genes identified and extent to which open reading frames were determinable. Sequence identification numbers are provided for nucleotide sequences as well as deduced amino acid sequences where located.

Table 2
A. pleuropneumoniae Open Reading Frames

Complete Open Reading Frame		NO Start Codon - Stop Codon	
atpH	SEQ ID NO: 134	dksA	SEQ ID NO: 136
aptG	SEQ ID NO: 132	dnaK	SEQ ID NO: 138
exbB	SEQ ID NO: 140	HI0379	SEQ ID NO: 144
OmpP5	SEQ ID NO: 152		
OmpP5-2	SEQ ID NO: 150	NO Start Codon - NO Stop Codon	
tig	SEQ ID NO: 160	pnp	SEQ ID NO: 154
fkpA	SEQ ID NO: 142	apvA-or 1	SEQ ID NO: 122
hupA	SEQ ID NO: 146	apvA-or 2	SEQ ID NO: 124
rpmF	SEQ ID NO: 158	apvB	SEQ ID NO: 126
		apvD	SEQ ID NO: 130
Start Codon - NO Stop Codon			
lpdA	SEQ ID NO: 148	RNA or Noncoding Sequences	
potD	SEQ ID NO: 156	tRNA-leu	SEQ ID NO: 162
yaeE	SEQ ID NO: 164	tRNA-glu	SEQ ID NO: 163
apvC	SEQ ID NO: 128		

The putative identities listed in Table 3 (below, Example 9) were assigned by comparison with bacterial databases. The 110 mutants represented 35 groups of unique transposon insertions. The number of different mutations per loci varied, with some clones always containing an insertion at a single site within an ORF to clones containing insertions within different sites of the same ORF. Three multiple insertions were detected in the 110 mutants screened as determined by production of multiple PCR bands and generation of multiple sequence electropherograms.

Example 9

Competition Challenge of *A. pleuropneumoniae* Mutants with Wild Type APP225

A representative clone from each of the unique attenuated mutant groups identified above that was absent or highly reduced in the recovered population was isolated from the original pool plate and used in a competition challenge experiment with the wild type strain (AP225) to verify the relative attenuation caused by the transposon mutation. Mutant and wild type strains were grown in BHIV[10] to an OD_{590} of 0.6 – 0.9. Approximately 5.0 x 10^6 CFU each of the wild type and mutant strains were added to 4 mls BHI. The total 4 ml dose was used infect a 10-20 kg SPF pig by intratracheal

administration with a tracheal tube. At approximately 20 hours post-infection, all surviving animals were euthanized and the lungs removed. Lung lavages were performed as described above. Plate counts were carried out on BHIV^{10}N^{50} and BHIV^{10}N^{50}K^{100} to determine the relative numbers of wild type to mutant in both the input cultures and in the lung lavage samples. A Competitive Index (CI) was calculated as the [mutant CFU / wild type CFU]$_{input}$ / [mutant CFU / wild type CFU]$_{recovered}$.

Of the 35 potential transposon mutants, 22 were significantly attenuated, having a competitive index (CI) of less than 0.2. A transposon mutant that did not seem to be attenuated based on the STM screening results was chosen from one of the pools as a positive control. This mutant had a CI in vivo of approximately 0.6. An in vitro competition was also done for this mutant resulting in a CI of 0.8. The mutant was subsequently determined to contain an insertion between 2 phenylalanine tRNA's.

Competitive indices for unique attenuated single-insertion mutants are listed in Table 3. Competitive indices for *atpG*, *pnp*, and *exbB* App mutants indicated that the mutants were unable to compete effectively with the wild type strains and were therefore attenuated.

Table 3
Virulence and Proposed Function of *A. pleuropneumoniae* Mutants

Mutant	Similarity	Putative or Known Functions	C.I.
AP20A6	*atpH*	ATP synthase	.009
AP7F10	*atpG*	ATP synthase	.013
AP17C6	*lpdA*	dihydrolipoamide dehydrogenase	.039
AP11E7	exbB	transport of iron compounds	.003,.003,.006
AP3H7	*potD*	Spermidine/putrescine transport	.308
AP8H6	*OmpP5*	Adhesin / OmpA homolog	.184
AP18H8	*OmpP5-2*	Adhesin / OmpA homolog	.552
AP13E9	*tig*	Peptidyl-prolyl isomerase	.050
AP13C2	*fkpA*	Peptidyl-prolyl isomerase	<.001
AP15C11	*pnp*	Polynucleotide phosphorylase	.032
AP18F12	*hupA*	Histone – like protein	.001
AP20F8	*dksA*	Dosage dependent suppressor of dnaK mutations	.075
AP5G4	*dnaK*	Heat shock protein – molecular chaperone	.376

AP17C9	*tRNA-leu*	Protein Synthesis (gene regulation?)	.059
AP5D6	*tRNA-glu*	Protein Synthesis	.055
AP18B2	*rpmF*	Protein Synthesis	.112
AP10E7	*yaeA*	Unknown	.001
AP19A5	HI0379	Unknown	.061
AP10C10	*apvA*	Unknown	.157
AP18F5	*apvB*	Unknown	.103
AP2A6	*apvC*	Unknown	.091
AP2C11	*apvD*	Unknown	.014

Accuracy of the CI appeared to be very good as the *exbB* mutant was competed within three different animals yielding CI's of 0.003, 0.003 and 0.006. The use of a Competitive Index number to assign attenuation based upon one competition in a large animal study was further confirmed based on preliminary vaccination results in pigs with 7 mutants (n=8) described below in Example 11.

Example 10
Characterization of Attenuated *A. pleuropneumoniae* Virulence Genes

The *A. pleuropneumoniae* genes identified represent four broad functional classes: biosynthetic enzymes, cellular transport components, cellular regulation components and unknowns.

The *atpG* gene, encoding the F1-γ subunit of the F_0F_1 H+-ATPase complex, can function in production of ATP or in the transport of protons by hydrolyzing ATP. A related *atpG* attenuated mutant was also identified in *P. multocida*. Another *atp* gene, *atpH*, that encodes the F_1 δ subunit was also identified. Phenotypes of *atp* mutants include non-adaptable acid-sensitivity phenotype [Foster, *J Bacteriol. 173*:6896-6902 (1991)], loss of virulence in *Salmonella typhimurium* [Garcia del Portillo, *et al., Infect Immun. 61*:4489-4492 (1993)] and *P. multocida* (above) and a reduction in both transformation frequencies and induction of competence regulatory genes in *Haemophilus influenzae* Rd [Gwinn, *et al., J Bacteriol. 179*:7315-20 (1997)].

LpdA is a dihydrolipoamide dehydrogenase that is a component of two enzymatic complexes: pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase.

While the relationship to virulence is unknown, production of LpdA is induced in *Salmonella typhimurium* when exposed to a bactericidal protein from human which may suggest that this induction may be involved in attempts to repair the outer membrane [Qi, *et al., Mol Microbiol. 17*:523-31 (1995)].

Transport of scarce compounds necessary for growth and survival are critical in vivo. ExbB is a part of the TonB transport complex [Hantke, and Zimmerman, *Microbiology Letters. 49*:31-35 (1981)], interacting with TonB in at least two distinct ways [Karlsson, *et al., Mol Microbiol. 8*:389-96 (1993), Karlsson, *et al., Mol Microbiol. 8*:379-88 (1993)]. Iron acquisition is essential for pathogens. In this work, attenuated *exbB* mutants in both APP and *P. multocida* have been identified. Several TonB-dependent iron receptors have been identified in other bacteria [Biswas, *et al., Mol. Microbiol. 24*:169-179 (1997), Braun, *FEMS Microbiol Rev. 16*:295-307 (1995), Elkins, *et al., Infect Immun. 66*:151-160 (1998), Occhino, *et al., Mol Microbiol. 29*:1493-507 (1998),Stojiljkovic and Srinivasan, *J Bacteriol. 179*:805-12 (1997)]. *A. pleuropneumoniae* produces 2 transferrin-binding proteins, which likely depend on the ExbB/ExbD/TonB system, for acquisition of iron. PotD is a periplasmic binding protein that is required for spermidine (a polyamine) transport [Kashiwagi, *et al., J Biol Chem. 268*:19358-63 (1993)]. Another member of the *Pasteurellaceae* family, *Pasteurella haemolytica*, contains a homologue of *potD* (Lpp38) that is a major immunogen in convalescent or outer membrane protein vaccinated calves [Pandher and Murphy, *Vet Microbiol. 51*:331-41 (1996)]. In *P. haemolytica*, PotD appeared to be associated with both the inner and outer membranes. The role of PotD in virulence or in relationship to protective antibodies is unknown although previous work has shown *potD* mutants of *Streptococcus pneumoniae* to be attenuated [Polissi, *et al., Infect. Immun. 66*:5620-9 (1998)].

Relatively few "classical virulence factors," such as adhesins or toxins with the exception of homologues to OMP P5 of *Haemophilus influenzae*, were identified. *H. influenzae* OMP P5 is a major outer membrane protein that is related to the OmpA porin family of proteins [Munson, *et al., M Infect Immun. 61*:4017-20 (1993)]. OMP P5 in nontypeable *Haemophilus influenzae* has been shown to encode a fimbrial subunit protein expressed as a filamentous structure [Sirakova, *et al., Infect Immun.*

62:2002-20 (1994)] that contributes to virulence and binding of both mucin and epithelial cells [Miyamoto and Bakaletz, *Microb Pathog. 21*:343-56 (1996), Reddy, *et al., Infect Immun. 64*:1477-9 (1996), Sirakova, *et al., Infect Immun. 62*:2002-20 (1994)]. A significant finding was identification of two distinct ORF's that appear to encode OMP P5 homologues. This is also the case with two very similar proteins, MOMP and OmpA2 from *Haemophilus ducreyi*. It remains to be determined whether both are functionally involved in the production of fimbriae and whether the presence of two such ORFs represents a divergent duplication with redundant or complementing functions. Interestingly, the two OMP P5 mutants seem to have disparate CI values, suggesting a difference in essentiality or functionality for only one copy. OMP P5 has been shown to undergo molecular variation during chronic infections [Duim, *et al., Infect Immun. 65*:1351-1356 (1997)], however, this appears to be restricted to a single gene undergoing point mutations resulting in amino acid changes rather than "type switching" due to differential expression of multiple genes.

Protein folding enzymes are important accessories for the efficient folding of periplasmic and extracellular proteins, and two genes were identified whose products have peptidyl-prolyl isomerase activity: *fkpA* and *tig* (trigger factor). FkpA is a periplasmic protein that is a member of the FK506-binding protein family [Horne and Young, *Arch Microbiol. 163*:357-65 (1995); Missiakas, *et al., Mol Microbiol. 21*:871-84 (1996)]. FkpA has been shown to contribute to intracellular survival of *Salmonella typhimurium* [Horne, *et al., Infect Immun. 65*:806-10 (1997)] and a *Legionella pneumophila* homolog, *mip* [Engleberg, *et al., Infect Immun. 57*:1263-1270 (1989)], is responsible for virulence and infection of macrophages [Cianciotto, *et al., J. Infect. Dis. 162*:121-6 (1990); Cianciotto, *et al., Infect. Immun. 57*:1255-1262 (1989)]. Tig, or trigger factor [Crooke and Wickner, *Proc. Natl. Acad. Sci. USA. 84*:5216-20 (1987), Guthrie, and Wickner, *J Bacteriol. 172*:5555-62 (1990), reviewed in Hesterkamp, and Bukau., *FEBS Lett. 389*:32-4 (1996)], is a peptidyl prolyl isomerase containing a typical FKBP region [Callebaut and Mornon, *FEBS Lett. 374*:211-215 (1995)], but is unaffected by FK506 [Stoller, *et al., EMBO J. 14*:4939-48 (1995)]. Tig has been shown to associate with the ribosomes and nascent polypeptide chains [Hesterkamp, *et al., Proc Natl Acad Sci USA 93*:4437-41 (1996), Stoller, *et al., EMBO J. 14*:4939-48 (1995)]. Possible roles

include an unknown influence on cell division [Guthrie, and Wickner, *J Bacteriol. 172*:5555-62 (1990)] in *E. coli*, a role in the secretion and activation of the *Streptococcus pyogenes* cysteine proteinase [Lyon, *et al., EMBO J. 17*:6263-75 (1998)] and survival under starvation conditions in *Bacillus subtilis* [Gothel, *et al., Biochemistry 37*:13392-9 (1998)].

Bacterial pathogens employ many mechanisms to coordinately regulate gene expression in order to survive a wide variety of environmental conditions within the host. Differences in mRNA stability can modulate gene expression in prokaryotes [Belasco and Higgins, *Gene 72*:15-23 (1988)]. For example, *rnr* (*vacB*) is required for expression of plasmid borne virulence genes in *Shigella flexneri* [Tobe, *et al., J Bacteriol. 174*:6359-67 (1992)] and encodes the RnaseR ribonuclease [Cheng, *et al., J. Biol. Chem. 273*:14077-14080 (1998)]. PNP is a polynucleotide phosphorylase that is involved in the degradation of mRNA. Null *pnp / rnr* mutants are lethal, suggesting a probable overlap of function. It therefore is possible that both *rnr* and *pnp* are involved in the regulation of virulence gene expression. A *pnp* mutant of *P. multocida* is avirulent in a mouse septicemic model (Example 2)]. Other *pnp*-associated phenotypes include competence deficiency and cold sensitivity in *Bacillus subtilis* [Wang and Bechhofer, *J Bacteriol. 178*:2375-82 (1996)].

HupA is a bacterial histone-like protein, which in combination with HupB constitute the HU protein in E. coli. Reports have suggested that *hupA* and *hupB* single mutants do not demonstrate any observable phenotype [Huisman, *et al., J Bacteriol. 171*:3704-12 (1989), Wada, *et al., J Mol Biol. 204*:581-91 (1988)], however, *hupA-hupB* double mutants have been shown to be cold sensitive, sensitive to heat shock and blocked in many forms of site-specific DNA recombination [Wada, *et al., J Mol Biol. 204*:581-91 (1988), Wada, *et al., Gene. 76*:345-52 (1989)]. One limited data previously indicated that *hupA* is directly involved in virulence [Turner, *et al., Infect Immun. 66*:2099-106 (1998)]. The mechanism of *hupA* attenuation remains unknown.

DnaK is a well known and highly conserved heat shock protein involved in regulatory responses to various stressful environmental changes [reviewed in Lindquist and Craig, *Annu Rev Genet. 22*:631-77 (1988)]. DnaK is also one of the most significantly induced stress proteins in *Yersinia enterocolitica* after being phagocytosed

by macrophages [Yamamoto, *et al., Microbiol Immunol. 38*:295-300 (1994)] and a *Brucella suis dnaK* mutant failed to multiply within human macrophage-like cells [Kohler, *et al., Mol Microbiol. 20*:701-12 (1996)]. In contrast, another intracellular pathogen, *Listeria monocytogenes*, did not show induction of *dnaK* after phagocytosis [Hanawa, *et al., Infect Immun. 63*:4595-9 (1995)]. A *dnaK* mutant of *Vibrio cholera* affected the production of ToxR and its regulated virulence factors in vitro but similar results were not obtained from in vivo grown cells [Chakrabarti, *et al., Infect Immun. 67*:1025-1033 (1999)]. The CI of *A. pleuropneumonia dnaK* mutant was higher than most of the attenuated mutants although still approximately half of the positive control strain.

DksA is a dosage dependent suppressor of filamentous and temperature-sensitive growth in a *dnaK* mutant of *E. coli* [Kang and Craig, *J Bacteriol. 172*:2055-64 (1990)]. There is currently no defined molecular function for DksA, but the gene has been identified as being critical for the virulence of *Salmonella typhimurium* in chickens and newly hatched chicks [Turner, *et al., Infect Immun. 66*:2099-106 (1998)]. In that work, it was noted that the *dksA* mutant did not grow well with glucose or histidine but did grow well with glutamine or glutamate as the sole carbon source. This observation may indicate that the *dksA* mutant is somehow impaired in the biosynthesis of glutamate [Turner, *et al., Infect Immun. 66*:2099-106 (1998)].

Three genes were identified that have roles in protein synthesis: tRNA-leu, tRNA-*glu* and *rpmF*. Excluding protein synthesis, tRNA's also have a wide variety of functional roles in peptidoglycan synthesis [Stewart, *et al., Nature 230*:36-38 (1971)], porphyrin ring synthesis [Jahn, *et al., Trends Biochem Sci. 17*:215-8 (1992)], targeting of proteins for degradation [Tobias, *et al., Science 254*:1374-7 (1991)], post-translational addition of amino acids to proteins [Leibowitz and Soffer, *B.B.R.C. 36*:47-53 (1969)] and mediation of bacterial-eukaryotic interactions [Gray, *et al., J Bacteriol. 174*:1086-98 (1992), Hromockyj, *et al., Mol Microbiol. 6*:2113-24 (1992)]. More specifically, tRNA-leu is implicated in transcription attenuation [Carter, *et al., Proc. Natl. Acad. Sci. USA 83*:8127-8131 (1986)], lesion formation by *Pseudomonas syringae* [Rich and Willis, J Bacteriol. 179:2247-58 (1997)] and virulence of uropathogenic *E. coli* [Dobrindt, *et al., FEMS Microbiol Lett. 162*:135-141 (1998), Ritter, *et al., Mol Microbiol. 17*:109-21

(1995)]. It is unknown whether the tRNA that we have identified represents a minor species of tRNA-leu in *A. pleuropneumoniae*. Regardless, it is possible that tRNA-leu may have any one of a wide range of functions. RpmF is a ribosomal protein whose gene is also part of an operon containing fatty acid biosynthesis enzymes in *E. coli*. Further work will be required to indicate if this is the case in *A. pleuropneumoniae*, although the same clustering of *fab* genes and *rpmF* occurs in *Haemophilus influenzae* [Fleischmann, *et al., Science 269*:496-512 (1995)]. The expression of the *fab* genes is not necessarily dependent on transcripts originating upstream of *rpmF* as there has been a secondary promoter identified within *rpmF* [Zhang and Cronan, Jr., *J Bacteriol. 180*:3295-303 (1998)].

The final class of attenuated mutants includes mutations within genes of unknown function or genes that have not been previously identified. Homologs of *yaeA* and HI0379 have previously been identified in *Escherichia coli* [Blattner, *et al., Science 277*:1453-1474 (1997)] and *Haemophilus influenzae* [Fleischmann, *et al., Science 269*:496-512 (1995)], respectively. The remaining unknowns have been designated Actinobacillus pleuropneumoniae virulence genes (apv). The *apvC* gene shows significant similarity to HI0893, however, the proposed similarity of HI0893 as a transcriptional repressor similar to the fatty acid response regulator Bm3R1 [Palmer, *J Biol Chem. 273*:18109-16 (1998)] is doubtful. The *apvD* gene is also most similar to a putative membrane protein (b0878) with unknown function from *E. coli* [Blattner, *et al., Science 277*:1453-1474 (1997)]. Two other unknowns, *apvA* and *apvB* had no significant matches in the public databases.

Example 11
Safety and Efficacy of *A. pleuropneumoniae* Mutants

Nine groups (n=8) of SPF pigs (4-5 weeks old, 3-10 kg) were used to determine the safety and efficacy of seven *A. pleuropneumoniae* mutants as live attenuated vaccine strains. Seven groups were infected intranasally with 10^{10} CFU of each mutant on day 1. One group was vaccinated on days 1 and 15 with the commercially available vaccine Pleuromune (Bayer), and one naive group was not vaccinated. On day 29, all groups were challenged intranaslally with 1-5 x 10^5 CFU per

pig of wild type APP225. All surviving animals were euthanized and necropsied on day 42 of the study. Results are shown in Table 4.

Table 4
Efficacy of *A. pleuropneumoniae* Mutants

Vaccine	% Mortality following intranasal challenge	
	Vaccination	Challenge
Pleuromune	0	37.5
exbB	0	0
tig	12.5	0
fkpA	12.5	0
HI0385	50.0	0
pnp	0	0
yaeE	0	0
atpG	0	0
None	N/A	50.0

The *exbB*, *atpG*, *pnp*, and *yaeA* mutants caused no mortality when administered at a dosage of 10^{10} CFU intranasally. The *fkpA* and *tig* mutant groups had one death each and the HI0379 group (highest April 6, 2000CI of the 7 mutants tested shown in Example 9) had four deaths. Wildtype LD_{50} using this model was generally 1 x 10^7 CFU, indicating that each of these mutants is at least 100 fold attenuated and that there is a reasonable correlation between CI and attenuation.

Numerous modifications and variations in the invention as set forth in the above illustrative examples are expected to occur to those skilled in the art. Consequently only such limitations as appear in the appended claims should be placed on the invention.